The Human Leukocyte Antigen G as an Immune Escape Mechanism and Novel Therapeutic Target in Urological Tumors

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The non-classical human leukocyte antigen G (HLA-G) is a potent regulatory protein involved in the induction of immunological tolerance. This is based on the binding of membrane-bound as well as soluble HLA-G to inhibitory receptors expressed on various immune effector cells, in particular NK cells and T cells, leading to their attenuated functions. Despite its restricted expression on immune-privileged tissues under physiological conditions, HLA-G expression has been frequently detected in solid and hematopoietic malignancies including urological cancers, such as renal cell and urothelial bladder carcinoma and has been associated with progression of urological cancers and poor outcome of patients: HLA-G expression protects tumor cells from anti-tumor immunity upon interaction with its inhibitory receptors by modulating both the phenotype and function of immune cells leading to immune evasion. This review will discuss the expression, regulation, functional and clinical relevance of HLA-G expression in urological tumors as well as its use as a putative biomarker and/or potential therapeutic target for the treatment of renal cell carcinoma as well as urothelial bladder cancer.

Keywords: HLA-G, renal cell carcinoma, epithelial bladder cancer, immune evasion, immunotherapy, immune cell infiltration

INTRODUCTION

During the last two decades it has been generally accepted that altered immune responses and immune evasion strategies are characteristic hallmarks of cancer. It has been demonstrated that the immune system within a tumor undergoes changes in its cellular composition, functionality and localization (1). While effector cells are often precluded from the invasive margin of tumors, immune suppressive effector cells are frequently found in this localization. Tumor extrinsic factors, like immune suppressive cells, soluble immune modulatory molecules, e.g. prostaglandin, arginase, metabolites or (anti-inflammatory) cytokines, will alter either the composition or the activity of

tumor infiltrating lymphocytes (TILs) and promote tumor growth and metastasis (2). Further changes of the tumor microenvironment (TME) include an altered metabolism resulting in a low pH, hypoxia and chronic inflammation, which are predisposing factors and implicated in modulating the immune cell repertoire and contributing to immunological dysfunction (3, 4). Defects of immune sensing mediated by the expression of inhibitory immune checkpoint receptors (ICP-R), such as e.g. the program death-1 receptor (PD-1), the CTL-associated antigen-4 (CTLA-4), T cell immunoreceptor with immunoglobulin and ITIM domain (TIGIT), T cell immunoglobulin 3 (TIM-3), V-domain Ig suppressor of T cell activation (VISTA) and the lymphocyte activation gene (LAG-3) expressed on T and/or natural killer (NK) cells, represent so far known major immune escape mechanisms (5, 6). Thus, there is an urgent need for appropriate strategies in order to revert the immune suppressive TME to a more stimulatory milieu. In addition, tumor cells themselves are constantly developing strategies to escape immune surveillance, e.g. by altering the expression of classical and non-classical human leukocyte antigens (HLAs) and immune checkpoint ligands (ICP-L) and/or ICP-R (7). This will also lead to a reduced reactivity to innate and adaptive immune responses. Thus, a plethora of distinct features of tumor and immune cells limit the treatment efficacy and clinical outcome to cancer (immuno)therapies.

Novel cancer immunotherapies, such as immune checkpoint (ICP) inhibitors and adoptive cell therapy, have been developed during the last decade and demonstrated a therapeutic efficacy in hematologic tumors, but also in some solid cancers. However, the response rates to these therapies, in particular to solid tumors, still need to be improved and only a few patients achieve durable response rates due to intrinsic and/or acquired resistances mediated by immune evasion strategies of the tumor cells. This could be driven by both cellular and molecular suppressive networks within the TME, but also within the tumor, such as e.g. the loss of tumor antigens, downregulated expression of HLA class I molecules and antigen processing machinery (APM) and interferon (IFN) pathway components as

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Abbreviations: AIRE, autoimmune regulator; APC, antigen presenting cell; APM, antigen processing machinery; β2-m, β2-microglobulin; BC, bladder carcinoma; BCG, Bacillus Calmette-Guerin; bp, base pair; CAR, chimeric antigen receptor; ccRCC, clear cell renal cell carcinoma; CRC, colorectal carcinoma; CREP1, cAMP-responsive element binding protein 1; CTL, cytotoxic T lymphocyte; CTLA4, cytotoxic T lymphocyte-associated protein-4; DC, dendritic cell; EV, extracellular vesicle; HC, heavy chain; HIF, hypoxia inducible factor; HLA, human leukocyte antigen; ICP, immune checkpoint; ICP-L, immune checkpoint ligand; ICP-R, immune checkpoint receptor; IFN, interferon; IHC, immunohistochemistry; ILT, immunoglobulin-like transcript; IL-10; LAG3, lymphocyte activation gene 3; mAb, monoclonal antibody; MHC, major histocompatibility complex; miRNA, microRNA; MMP2, matrix metalloproteinase 2; NK, natural killer; OS, overall survival; PD1, programmed cell death-1; PD-L1, programmed death ligand 1; PFS, progression-free survival; RBP, RNA-binding protein; RCC, renal cell carcinoma; sHLA-G, soluble HLA-G; TAP, transporter associated with antigen processing; TGF-β, transforming growth factor β; TIGIT, T cell immune receptor with immunoglobulin and ITIM domain; TIL, tumor infiltrating lymphocyte; TIM-3, T cell immunoglobulin and mucin domain 3; TM, transmembrane; TME, tumor microenvironment; Treg, regulatory T cell; UTR, untranslated region; VEGF, vascular endothelial growth factor; VHL, Von Hippel-Lindau; VISTA, V-domain Ig suppressor of T cell activation; WB, Western blot.

well as upregulation of various immune checkpoint (ICP) molecules, like PD-L1, B7-H4, B7-H6, LAG-3, TIM-3, VISTA, HLA-E or HLA-G (8–10).

GENERAL FEATURES OF HLA-G - GENE STRUCTURE, EXPRESSION, REGULATION, PHYSIOLOGIC FUNCTION

HLA-G is a non-classical HLA class Ib molecule with a length of 4144 base pairs (bp) and is localized like the classical HLA class Ia molecules within the cluster of the major histocompatibility complex (MHC) on the short arm of chromosome 6 at region 6p21.3. However, it has distinct properties to HLA class Ia molecules (HLA-A, -B, -C) such as a highly restricted and tightly regulated expression, which is under physiological conditions limited to immune-privileged tissues/organs with confined local immune and inflammatory responses (11) preventing irreversible tissue damages. HLA-G expression was found on the cornea, but also on conjunctival and retinal pigment epithelial cells (12–14), insulin- and glucagon-positive cells within the endocrine islets of the pancreas (15), medullary thymic epithelial cells (16) and on extravillous trophoblasts of the placenta (17–19) protecting the fetus with its paternal antigens from maternal immune rejection (20–22). In these tissues, HLA-G contributes to immunological tolerance by acting as a ligand to inhibitory receptors expressed on several immune effector cells (23). Thus, HLA-G belongs to the few immunomodulatory proteins, whose main function lies in the mediation of a sufficient immunological tolerance even to foreign antigens. Although HLA-G is not physiologically expressed in most adult tissues, neoexpression/(re)expression of HLA-G is a frequently observed phenomenon in different cancers thereby inducing an immunological tolerance and suppression of immune surveillance (18, 19, 24). Due to alternative splicing, the HLA-G protein can exist as membrane-bound isoforms and soluble forms (25–27), which bind to inhibitory receptors of immune effector cells thereby inhibiting immune responses (28, 29): (i) leukocyte immunoglobulin-like receptor, subfamily B, member 1/LILRB1 (synonym: Ig-like transcript 2/ILT2; CD85J), (ii) leukocyte immunoglobulin-like receptor, subfamily B, member 2/LILRB2 (synonym: Ig-like transcript 4/ILT4; CD85D), (iii) killer cell immunoglobulin-like receptor, two domains long cytoplasmic tail, 4/KIR2DL4 (CD158D), (iv) killer cell lectin-like receptor, subfamily c, member 1/KLRC1 (synonym: natural killer cell lectin; NKG2A) and (v) natural killer cell receptor By55 (CD160). LILRB1 is expressed on monocytes, dendritic cells (DCs), B cells, NK cells and T cells; LILRB2 on cells of myeloid origin, KIR2DL4 on NK cells, KLRC1 on approximately 50% of NK cells and on a subset of CD8$^+$ T cells and CD160 is expressed on NK cells. Furthermore, HLA-G can be expressed and secreted from non-cancer cells, such as human mesenchymal stem cells (hMSCs). These hMSCs can inhibit both NK cell cytotoxicity and T lymphocyte alloproliferation (30). In addition, the HLA-G receptor LILRB2

(ILT4) has been shown to be expressed on hematopoietic stem cells supporting their stemness through binding to angiopoietin-like proteins (29).

The expression of LILRB1/ILT2 and LILRB2/ILT4 on different immune effector cells, endothelial cells and tumor cells and the effects of the interaction with HLA-G have been recently summarized by Carosella and co-authors (31), while the different affinities of the HLA-G receptors to HLA-G as ligand and the exact binding positions have been reviewed elsewhere (26).

Another different feature of HLA-G compared to the HLA class Ia molecules is the low number of HLA-G alleles. To date (October 2021) the IPD and IMGT/HLA database (32) enlists 88 different HLA-G alleles compared to the several thousands of different HLA-A/B/C alleles. Furthermore, alternative splicing is commonly found within the human *HLA-G* gene leading to at least seven different HLA-G protein encoding splice variants (HLA-G1-HLA-G7) reviewed by Hviid, 2006 (33). Next to the primary transcript of HLA-G (HLA-G1), containing the α-1, -2 and -3 domains as well as a transmembrane (TM) domain resulting in a membrane-bound protein, several other membrane-bound and soluble HLA-G isoforms have been described (34–37) (**Table 1**).

While the isoforms HLA-G1 to HLA-G4 encode for membrane-bound proteins, the isoforms HLA-G5 to HLA-G6 contain the unspliced intron 4, HLA-G7 the unspliced intron 2 resulting in an early stop codon and leading to the loss of the transmembrane domain of HLA-G. As a consequence, the proteins HLA-G5 to HLA-G7 are soluble and secreted thereby contributing to changes in the local and peripheral microenvironment (38). The most abundant HLA-G proteins are HLA-G1 and HLA-G5, which both consist of a heavy chain with three domains (α1, α2, α3). In both cases the heavy chains are associated with the β2-microglobulin (β2-m) and these complexes can even present a limited and cell type-specific peptide repertoire towards CD8^{+} cytotoxic T lymphocytes (CTL) (39), which is not a prerequisite for their inhibitory functions towards immune effector cells.

In contrast, the other HLA-G isoforms are not bound to β2-m lacking one or two α domains, e.g. HLA-G2 (has α1 and α3), HLA-G3 (α1), HLA-G4 (α1 and α2), HLA-G6 (α1 and α3), and HLA-G7 (α1) (33). Not only the alternative splicing, but also the proteolytic shedding of membrane-bound HLA-G proteins mediated by the matrix metalloproteinase (MMP) 2 leads to the generation of sHLA-G protein isoforms (40, 41). It is noteworthy that in addition to the functional role of membrane-bound and soluble HLA-G, the knowledge of the splicing pattern must be considered for the choice of respective antibodies for quantification and/or identification of the cellular localization. Recently, a number of antibodies directed against the different HLA-G isoforms have become available for appropriate protein detection and quantification as summarized by Krijgsman and co-authors (42). Their use in combination with antibodies staining HLA-G receptors demonstrated a heterogeneous expression leading to a fine-tuned network regulating the HLA-G-mediated immune interaction (42).

HLA-G expression is tightly regulated at different levels (**Figure 1**), such as transcriptional, posttranscriptional as well as epigenetic control. Indeed, a HLA-G promoter methylation can be frequently found in HLA-G-negative cells, which could be reversed by demethylating agents thereby inducing HLA-G transcription (43, 44). DNA methylation of its promoter is based on the covalent binding of a methyl (-CH3) group to a cytosine residue in CpG dinucleotides enriched CpG islands (45). The degree of CpG methylation at the HLA-G promoter is associated with HLA-G expression (46). Chromatin immunoprecipitation demonstrated a differential histone acetylation status of HLA-G[+]-expressing vs. HLA-G-non-expressing cells. Treatment with histone deacetylation inhibitors (HDACi), like sodium butyrate or trichostatin A (TSA), induced histone hyperacetylation, which was associated with a reversion of HLA-G repression (47). In addition to epigenetic mechanisms, HLA-G expression is transcriptionally regulated. In this context, several HLA-G relevant transcription factors have been reported. In the HLA-G expressing medullary thymic epithelial cells, the transcription factor AIRE has been identified to increase HLA-G transcription (48), but it has to be mentioned that the AIRE expression dramatically decreases by age-related thymic involution (49, 50), which could affect indirectly the HLA-G expression. Recently, the cAMP-responsive element binding protein (CREB)1 and IRF1 have been demonstrated to bind HLA-G promoter sequences leading to an increased HLA-G transcription (51–53). In contrast, RREB-1 and LINE1 repressed HLA-G gene transcription (54, 55). Furthermore, several stress stimuli have been reported to increase HLA-G transcription, including heat stress, heavy metal stress, viral/bacterial/parasitic infections [as reviewed by L. Amiot (56)] and several cytokines, such as IL-10, IL-6 and IFN-γ (46, 57, 58).

TABLE 1 | Overview of all described HLA-G isoforms.

HLA-G isoforms	Alternative Splicing	Effect	Reference
HLA-G1	wild type	membrane-bound heavy chain (HC) with α-1, α-2, α-3 domain and transmembrane domain (TM)	(34–36)
HLA-G2	no exon 3	membrane-bound lack of α-2	(34)
HLA-G3	no exon 3 and 4	membrane-bound lack of α-2 and α-3	(34)
HLA-G4	no exon 4	lack of α-3	(34)
HLA-G5	includes intron 4 translation stops after exon 4	no TM domain; soluble; HC with α-1, α-2, α-3	(35)
HLA-G6	includes intron 4 translation stops after exon 4 no exon 3	no TM domain; soluble; lack of α-2	(35)
HLA-G7	includes intron 2 translation stops after exon 1	no TM domain; soluble; lack of α-2 and -3	(35)
HLA-G1L	5 additional N-terminal amino-acids (MKTPR)	membrane-bound	(36)

TM, transmembrane domain; intron 5 was previously known as intron 4 according to the IMGT/HLA nomenclature.
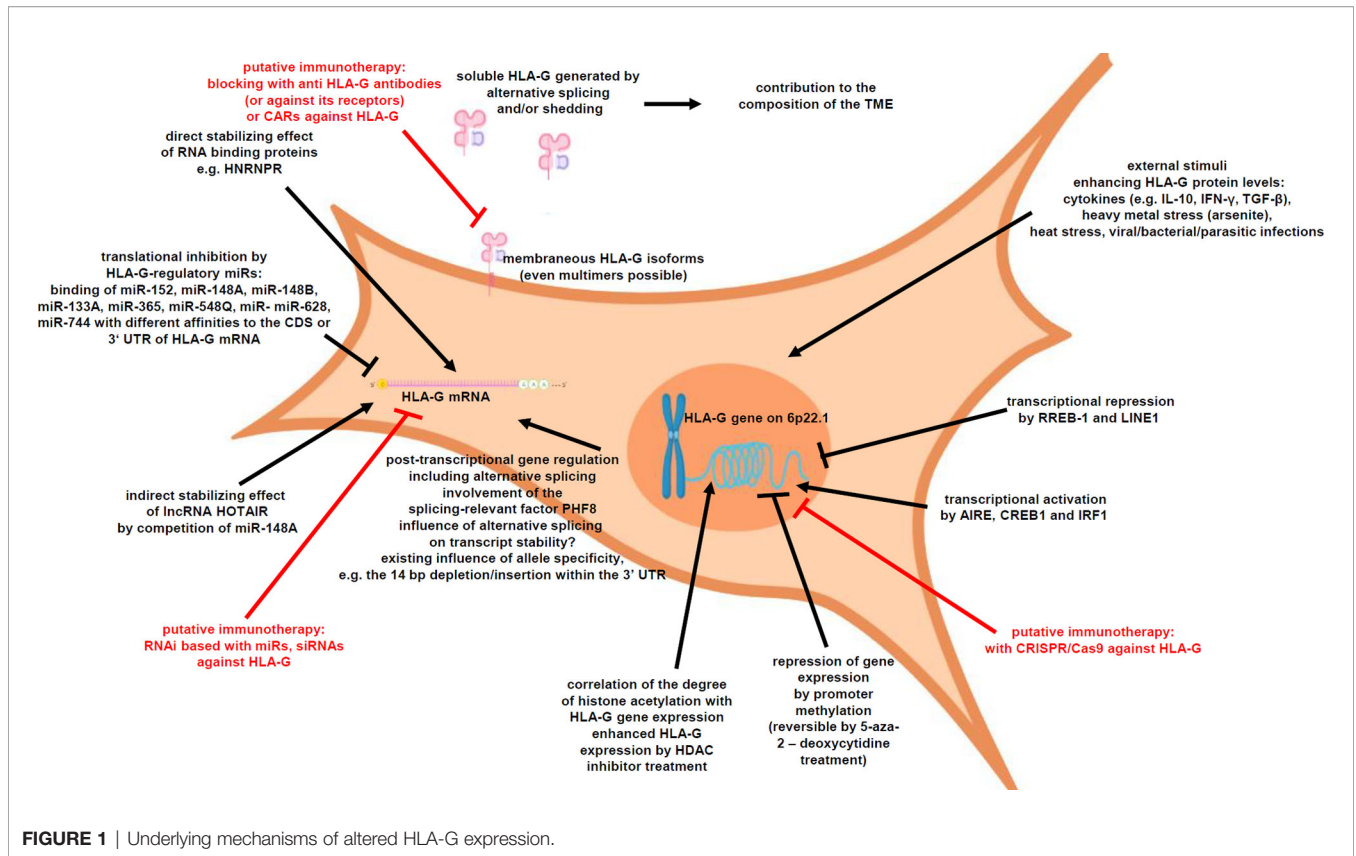
FIGURE 1 | Underlying mechanisms of altered HLA-G expression.

Next to the transcriptional regulation, HLA-G expression is posttranscriptionally controlled by microRNAs (miRNAs), RNA-binding proteins (RBPs) and long non-coding RNAs (lncRNAs) (59–61). A number of miRNAs have been reported to directly bind to the HLA-G mRNA and interfere with the HLA-G translation leading to HLA-G mRNA decay: miR-152, miR-148A, miR-148B, miR-133A, miR-365, miR-548q, miR-628, and miR-744 (57, 62–66). These miRNAs usually bind either to the 3'-untranslated region (UTR) or in the case of miR-744 to the coding sequence (CDS) of HLA-G. An allele specificity is the 14 nt deletion/insertion within the 3'-UTR of the HLA-G mRNA in regard to the binding of regulatory factors including miRNAs (67). Recently, a distinctive site only present in the 3' UTR of HLA-G was identified, which binds RBPs and miRNAs (68). In addition, the RBP HNRNPR has been identified to regulate HLA-G, but also classical HLA class I antigens (60), while the lncRNA HOTAIR modulates HLA-G expression by competitively binding the HLA-G regulatory miR-148A and miR-152 (66, 69).

HLA-G EXPRESSION IN SOLID AND HEMATOPOIETIC TUMORS AND ITS CLINICAL RELEVANCE

It has been recently demonstrated that HLA-G is crucial for tumor immune evasion and is also associated with malignant transformation (29). A pathophysiological expression of HLA-G

was frequently detected on the cell surface of solid and hematopoietic malignancies (70). These include breast cancer, non-small cell lung cancer, esophageal squamous cell carcinoma, gastric cancer, colorectal cancer, hepatocellular carcinoma, oral squamous cell carcinoma, cervical cancer, ovarian cancer, bladder cancer, pancreatic cancer, glioma, renal cell carcinoma, and thyroid cancer as well as leukemia and lymphoma (**Table 2**). In contrast, HLA-G expression in adjacent healthy tissues has not been detected. Next to membranous HLA-G expression, sHLA-G isoforms were often elevated in plasma or serum samples of tumor patients (85, 86). In addition, HLA-G has been detected in extracellular vesicles (EV) in the supernatant of tumor cells including melanoma cells and might play a role in cancer immune escape by inhibiting immune cells in the TME even at distal sites (87).

Since HLA-G is frequently associated with an advanced tumor stage and a poor prognosis of patients, a diagnostic and prognostic potential has been suggested for HLA-G in different cancer types.

FREQUENCY OF HLA-G EXPRESSION IN RENAL CELL CARCINOMA

Characteristic Features of RCC

Renal cell carcinoma (RCC) is the most common kidney tumor with an incidence of 2% of tumors in the Western world (88). Based on the histology several RCC subtypes were classified, with

TABLE 2 | Summary of pathological HLA-G neoexpression in solid and hematopoietic malignancies.

Tumor Entity	Cell Lines/tumor Samples/ Plasma Samples; Number of Samples	Method/Applied Antibody	Frequency of HLA-G Expression	Correlation of the HLA-G with Clinical Parameters	References
breast cancer	235 primary breast cancer lesions; 44 plasma samples of breast cancer patients and 48 plasma samples of healthy controls	IHC (mAb HGY), ELISA	66% HLA-G positive breast cancers; sHLA-G: 0.74 µg/mL in stage I patients, sHLA-G: 0.78 µg/mL in stage II patients; sHLA-G: 0.43 µg/mL in healthy donors	statistically significant correlation tumor size ($p = 0.0001$), nodal status ($p = 0.012$), disease stage ($p = 0.0001$), HLA-G positive patients with lower survival rate ($p < 0.028$); elevated sHLA-G levels in plasma of breast cancer patients ($p < 0.001$)	(71)
breast cancer	677 early breast cancer lesions	IHC (mAb 4H84)	60% HLA-G positive breast cancers	predictor for breast cancer patients	(72)
cervical cancer	22 normal cervical tissues, 14 cervical intraepithelial neoplasia patients, 129 patients with squamous cell cervical cancer	IHC (mAb 4H84)	0% in normal cervical tissues, 35.7% in cervical intraepithelial neoplasia, 62.8% in squamous cell cervical cancer patients	association with disease progression	(73)
cervical cancer	22 normal cervical tissues, 119 primary cervical lesions; 172 plasma samples of patients with cervical cancer and 20 plasma samples of healthy controls	IHC (mAb 4H84), ELISA (MEM-G/9)	0% in normal cervical tissues, 45% in primary cervical lesions; statistically significant higher sHLA-G levels in plasma of cervical patients (median 191.4 U/ml) versus plasma of healthy controls (median 45.18 U/ml, $p < 0.001$)	significant correlation ($p < 0.05$) to size of the main lesion, parametrical invasion and lymph node metastasis	(74)
colorectal cancer (CRC)	457 primary colorectal cancer (CRC) (colon = 232, rectal = 225 lesions)	IHC (mAb 4H84)	70.7% HLA-G positive CRC specimen	significant association with worse prognosis ($p = 0.042$)	(75)
colorectal cancer	144 plasma samples of CRC patients, 60 plasma samples of healthy controls	ELISA (MEM-G/9)	statistically significant ($p < 0.01$) increased sHLA-G plasma levels in CRC patients (median 124.3 U/ml) than in healthy controls (median 25 U/ml)	no correlation	(76)
gastric cancer	94 unselected patients with gastric adenocarcinoma (stage I to III)	IHC (mAb, 4H84)	25.5% HLA-G positive gastric adenocarcinoma specimen	significant association with ($p < 0.0001$), worse survival	(77)
glioblastoma	108 glioblastoma specimen	IHC (mAb, MEM-G/02)	60.2% HLA-G positive glioblastoma specimen	negative effects on the survival rate	(78)
hepatocellular carcinoma	74 primary hepatocellular carcinoma specimen	IHC (mAb, 4H84)	31% HLA-G positive hepatocellular carcinoma specimen	no correlation	(79)
ovarian cancer	169 primary ovarian carcinoma lesions with type II, high grade serous and undifferentiated	IHC (mAb 4H84)	47.9% HLA-G positive primary ovarian cancer specimen	significant correlation with a favorable prognosis ($p = 0.038$)	(80)
ovarian cancer	33 primary ovarian carcinoma lesions, 13 normal ovarian tissues	IHC (mAb 4H84)	66.7% HLA-G positive primary ovarian cancer specimen, 0% of the normal ovarian tissues	protection from NK cell lysis (in vitro)	(81)
pancreatic carcinoma	42 primary pancreatic carcinoma specimen	IHC (mAb, 4H84)	66% HLA-G positive pancreatic carcinoma specimen	significant correlation with grade ($p=0.007$), stage ($p=0.038$) and poor prognosis	(79)
testicular cancer	34 primary testicular cancer patients	IHC (mAb 4H84)	20.6% HLA-G positive samples	no correlation	(82)
chronic myeloid leukemia	68 chronic myeloid leukemia	ELISA (sHLA-G1, HLA-G5)	association of sHLA-G with HLA-G alleles	reduced event free survival	(83)
chronic lymphocytic leukemia	45 chronic lymphocytic leukemia	flow cytometry (MEM-G/9)	1 – 12% positive	independent prognostic factor	(84)

clear cell (cc)RCC as major subtype with an incidence of 75 % of all RCCs, followed by papillary and chromophobe RCC with an incidence of 10 % and 5%, respectively and other subtypes accounting for less than 1 % (89). Hypertension, smoking, diabetes mellitus, chronic kidney diseases, kidney cysts, kidney transplantation are known risk factors for this disease with a clear gender imbalance (1 female patient to 1.8 male patients) (90). In addition, genetic predispositions, like mutations in tumor suppressor genes, e.g. the von Hippel Lindau (VHL) and PTEN gene, have been reported (88, 91).

During the last decades, the therapeutic options of RCC improved starting from unspecific cytokine treatment using high doses of IL-2 for lymphocyte activated killer cell generation (92), followed by application of receptor tyrosine kinase inhibitors, like sorafenib, sunitinib and axitinib, or the mTOR inhibitors temsirolimus and everolismus. More recently, therapeutic mAbs directed against ICP axes, namely the PD1 and PD-L1 axis (e.g. nivolumab, durvalumab) as well as the CTLA-4 and B7-1/B7-2 axis (e.g. ipilimumab) were developed and introduced into the treatment regimens. Actual research focuses on the identification of novel ICPs as well as the development of mAbs directed against LAG-3, TIM-3, TIGIT and other ICP-R and/or ICP-L for mono- or combination therapy (93).

It is noteworthy that HLA-G neoexpression and/or LILRB2/ILT4 expression in ccRCCs has been recently linked to aberrant expression of the vascular endothelial factor (VEGF)A and VEGFC (94). Thus, receptor tyrosine kinase inhibitors, which block predominantly the neoangiogenesis by inhibiting the tyrosine kinase domain of the VEGF receptors, might be used in combination with anti-HLA-G therapies.

Frequency of HLA-G Expression in RCC

While HLA-G expression could not be detected in healthy renal tissue, the pathophysiological HLA-G expression in RCC lesions ranged between 30 and 60% and was found at the cell surface as well as in the cytoplasm. Furthermore, the HLA-G plasma levels were statistically significant increase in RCC patients (**Table 3**). In ccRCC, a high frequency of HLA-G mRNA and protein expression has been described, which is age and sex independent, while in other RCC subtypes HLA-G expression was not detected. **Table 3** summarizes the frequency of HLA-G expression in RCCs, which was determined by different methods including immunohistochemistry (IHC), Western blot (WB) analysis and/ or PCR for HLA-G detection as well as its clinical relevance.

Omitting early reports applying only RCC cell lines, studies analyzing RCC patient cohorts could demonstrate a statistically significant correlation between HLA-G expression and higher tumor grading and staging in RCC patients using IHC and ELISA. These data suggest that HLA-G might be an interesting prognostic marker for RCC.

Furthermore, a link between the pathophysiologic HLA-G expression and the frequency and composition of the immune cell infiltration was reported (57). The HLA-G expression in RCC appears to be also associated with an altered immune cell repertoire in the TME. These TILs did not express CD25 and CD69 activation markers within the HLA-G positive group confirming the

hypothesis that HLA-G expression might contribute to the immune evasion of the tumor cells by inhibition of immune effector cells. Interestingly, the HLA-G receptors LILRB2/ILT4 were detected in stromal macrophages, plasma cells and infiltrating lymphocytes of RCC samples suggesting the presence of an immune-tolerant microenvironment.

Analysis of mRNA and protein levels revealed a discordant HLA-G mRNA and protein expression pattern frequently occurred suggesting a post-transcriptional regulation of HLA-G in this tumor entity (70, 98). Furthermore, a loss of HLA-G mRNA and cell surface expression of ccRCC cells was observed during cell culture, which might be explained by the absence of TFs modulated by the hypoxic microenvironment, the lack of cytokines, such as IFN-γ, IFN-α and IL-10, or an increasing promotor methylation (96).

HLA-G EXPRESSION IN BLADDER CANCER

Urothelial bladder cancer (BC) is the 10th most common tumor worldwide with a high incidence in the Western world. It is more prevalent in male than female (99). The initiation and progression of BC is a multifactorial process and comprises of immune surveillance, immune balance and immune escape (100). There exists accumulated evidence that BCs evade immune surveillance by modulating immune suppressive networks in the TME and upregulating co-inhibitory molecules like PD-L1 and HLA-G (101, 102). A heterogeneous pathophysiological neoexpression of HLA-G has been demonstrated in various stages of urothelial BC ranging from a frequency between 16.7 to 68 %, whereas adjacent normal urothelium lacks HLA-G expression (103, 104). In contrast, sHLA-G levels in serum of bladder cancer patients and healthy controls did not differ. Furthermore, higher levels of HLA-G transcripts than HLA-G protein were found in bladder cancer suggesting a posttranscriptional control comparable to that of RCC lesions. However, HLA-G-regulating mRNAs have not been yet investigated in bladder cancer (104).

In addition, repeated applications of Bacillus Calmette Guerin (BCG) can induce HLA-G neoexpression extrinsically leading to acquired resistance to further BCG-based instillation therapy (105). Few studies also demonstrated that patients with non-muscle-invasive urothelial carcinoma have an increased prevalence of peripheral blood T cells that are susceptible to HLA-G-mediated immunosuppression through co-expression of ILT2 and ILT4 (106). High peripheral prevalence of T helper cells and CTL expressing ILT2 is also associated with an increased risk of recurrence of non-muscle invasive urothelial carcinoma (107). However, no other published data currently exist on the significance of HLA-G in urothelial BC. Due to the specific immunological function of HLA-G and the overall sobering results of conventional ICP-oriented immunotherapies, further research on the role and potential therapeutic target ability of HLA-G in local and advanced stages of urothelial BC is required.

To best of our knowledge meaningful studies with sufficient BC or RCC patient cohort sizes in regard to elevated HLA-G

TABLE 3 | Frequency of HLA-G expression and its clinical relevance in RCCs.

Cell Lines/tumor Samples/plasma Samples; Number of Samples	Method/Applied Antibody	Frequency of HLA-G Expression	Clinical Relevance	Study
18 primary RCC lesions with adjacent renal tissue	IHC (4H84)	primary RCC: 61.1%	none	(95)
37 primary RCC lesions with adjacent renal tissue; 24 RCC cell lines and 8 autologous normal kidney cells	WB (mAbs MEM-G/9 and MEM-G/1) qPCR	primary RCC lesions: 27% RCC cell lines: 12.5% mRNA positive, RCC cell lines: 8.3% protein positive	none	(96)
14 RCC cell lines	WB (mAb 4H84), qPCR	mRNA positive: 57% protein positive: 43%	n.a.	(43)
109 primary RCC lesions, 34 adjacent tumor negative renal tissue, 16 plasma samples of RCC patients	IHC/WB (mAb 4H84); ELISA (MEM-G/9)	primary RCC lesions: 47.7% ccRCC: 49.5% chromophobe: 50% (n: 2/4) collecting duct RCC: 50% (n: 3/6) RCC sHLA-G in RCC patients: 39.5 U/ml normal controls: 19.2 U/ml (P = 0.002)	none	(97)
453 primary RCC lesions	IHC (mAb 4H84)	RCC samples: 49.9% membranous: 38.1% cytoplasmic expression	higher frequency of stronger cytoplasmic HLA-G staining in grade 3 tumors than lower grade tumors (p = 0.014)	(57)
33 plasma samples of RCC patients and healthy control group	ELISA (MEM-G/9)	sHLA-G levels in RCC (46.6 U/ml) than in HC (18.3 U/ml); (p = 0.41)	correlation of higher sHLA-G levels with advanced tumor stage and progression	Rodrigo et al., 2016 (DOI: 10.1200/JCO.2016.34.15_suppl.e16066 Journal of Clinical Oncology 34, no. 15_suppl)

n.a., not analyzed.

protein levels in urine samples and its suitability as a potential prognostic marker has not yet been performed.

INTRATUMORAL HETEROGENEITY OF HLA-G EXPRESSION IN RCC AND BC

A strong intratumoral heterogeneity exists in RCC for the expression of ICPs, such as PD-L1, B7-H3, PD-L2 and HLA-G, in primary RCC lesions with a highly variable HLA-G expression either between patients' tumor samples or at different areas within a tumor tissue (36, 108). Tronik-Le Roux and co-authors showed that ccRCC tumors were strongly, diffusely positive or negative using an antibody (ab) directed against the HLA-G alpha-1 domain (36). However, using an antibody specific for amino acids of intron 4 recognizing HLA-G without a transmembrane domain, IHC staining results were highly variable ranging from weak, negative to strong staining (36). Due to the assumption that the HLA-G α-1 domain is present in all HLA-G isoforms, the results of HLA-G expression would have been negative for patient tumors according to the α-1 specific antibody, but were strongly positive for the intron 4 (=5) specific antibody (36). Another study also showed intratumoral heterogeneity in ccRCC patients (108) with very heterogeneous staining pattern for HLA-G ranging between 37 – 70% of HLA-G throughout one tumor using IHC as well as for ILT4, which preferentially binds HLA-G. In contrast, a different RCC tumor lack HLA-G expression, but exhibit heterogeneous staining of ILT4. Interestingly, the HLA-G staining was inversely correlated to the PD-L1 staining (108). A larger study with 109 mixed RCC lesions demonstrated HLA-G expression in different RCC subtypes except for papillary RCC and control tissues. Soluble HLA-G in plasma of RCC patients showed higher expression compared to controls (97).

Immunohistochemical analysis of 75 primary bladder transitional cell carcinoma (TCC) lesions demonstrated a HLA-G expression in 51 of 75 tumors while the normal bladder lacks HLA-G expression. However, the expression varied from negative to 100% positive (103). A similar intratumoral heterogeneity of HLA-G expression was also shown in colorectal cancer (CRC) using different HLA-G specific antibodies (109).

CLINICAL RELEVANCE OF HLA-G EXPRESSION IN RCC AND BLADDER CANCER

Due to the frequent pathophysiological HLA-G expression in solid and hematopoietic tumor entities a clinical relevance has been suggested. In **Table 2**, selected studies investigating the HLA-G expression in solid tumor entities with meaningful patient cohort sizes are summarized and correlations to clinical parameters are highlighted.

As shown in **Table 2**, a correlation of HLA-G expression with disease progression, tumor size and in some cases also with prognosis of RCC exists. It is noteworthy that the expression intensity of the HLA-G protein had an impact on clinical relevant parameters, such as tumor staging/grading, disease progression and patients' survival. In addition to alternative splicing of HLA-G transcripts, deletions, insertions and nucleotide polymorphisms of the *HLA-G* gene have been described, which are important parameters for cancer and clinical correlations (110). In a study with 56 metastatic RCC, the 14 bp insertion/deletion polymorphism in the 3' UTR was analyzed (111). A trend towards better patients outcome was demonstrated in the presence of the homozygosity for the 14 bp deletion allele, while a better patients' survival for RCC with heterozygotic T/C vs. homozygotic T/T nucleotide polymorphism at p3003 was detected. Other allelic groups of HLA-G (G*0104 and G*0103) were found to be associated with the susceptibility to urinary bladder papillary transitional carcinoma (112). Next to the HLA-G isoform expression, it will be important to analyze, whether HLA-G isoforms are present as monomers, dimers or even as homotrimers (113) associated with or without β_2-m (20) and with distinct immune suppressive activities. In particular, for the HLA-G dimer an efficient inhibition of CD8$^+$ T cells and granzyme B was shown (114). The formation of HLA-G multimers again affects the antibody-based recognition, since some mAbs are detecting the respective HC, but only in complex with the β_2-m.

In summary, HLA-G has multiple splicing mechanisms leading to different HLA-G isoforms, which are not all detected with commonly used HLA-G antibodies. A recommendation could be to use variable HLA-G antibodies in order to detect most HLA-G isoforms and draw conclusions with clinical parameters. It will also be imperative to understand the function of the different HLA-G domains and subsequently of the HLA-G multimers. These data highlight that the HLA-G protein is an interesting target for therapy based attempts for its downregulation and to target tumor cells in analogy to the anti-PD-L1 mAbs.

ROLE OF HLA-G FOR CANCER IMMUNOTHERAPY

Due to its interactions with numerous immune effector cell populations, the HLA-G neoexpression has rekindled interest as an immune checkpoint inhibitor and suggested as a potential target. In healthy human renal tissues about 47% (+/- 12%) of the immune cells were CD3$^+$ T cells, divided in 44% CD4$^+$ and 56% CD8$^+$ T cells. About 10% of total immune cells were CD14$^+$ and CD16$^+$ myeloid cells. The frequency of NK and B cells in the kidney epithelium was 18.2% ± 10.5% and 1.4% ± 1.2%, respectively (115).

Furthermore, a large effect of the HLA-G expression significantly influenced on the immune cell infiltration of RCC. When compared to HLA-G-negative RCCs, HLA-G-positive RCCs had a statistically significant higher infiltration of CD3$^+$ and CD8$^+$ cells and a non-statistically significant higher number of CD4$^+$ and CD56$^+$ cells. However, the T cell activation markers CD69 and CD25 did not show a statistically significant difference between HLA-G-negative and HLA-G-positive RCC samples (57).

Based on these characteristics, HLA-G is postulated as a novel potential immune checkpoint in different malignancies (29) raising the question how HLA-G expression influences existing cancer immunotherapies including checkpoint inhibitors?

This question is of increasing interest and therefore currently addressed by various research groups independent of urological malignancies (31). This is due to the HLA-G-mediated inhibition of various immune effector cells of the innate and adaptive immune system.

ICPs physiologically protect against an overreaction of the immune system. Tumor cells use ICPs to escape from immune surveillance. ICPs are e.g., the programmed cell death protein 1 (PDCD-1, PD-1) expressed on T cells, its ligand CD274, the programmed cell death protein ligand 1 (PD-L1; B7H1) expressed on tumor cells and immune cells (T cells, B cells, DCs, NKs, macrophages), the cytotoxic T-lymphocyte-associated antigen (CTLA-4) expressed on T cells (T helper cells, cytotoxic T cells and regulatory T cells) and its immunological counterparts B7-1/B7-2 (CD80/CD86) expressed on antigen presenting cells (APCs: DCs, monocytes, macrophages and B lymphocytes). Several ICP inhibitors targeting CTLA4 and the PD1/PD-L1 axes have been recently approved by the EMA and/or the FDA. Their application can result in an activation of different immune cells accompanied by tumor cell elimination, leading to the remarkable success of this therapeutic approach in different cancers including RCC and BC (116–121). Recently, multiple ICPs are investigated as novel targets in experimental tumor models or in clinical trials, like LAG3, TIM-3, TIGIT, BTLA and/or agonists of the co-stimulatory receptors GITR, OX40, 41BB and ICOS (93).

Next to HLA-G and PD-L1 (B7-H1) *in silico* analyses of RCC TCGA data also identified B7-H3, B7-H5, HVEM, CD40, CD70 and ILT2 (on tumor cells) as putative novel ICP axes (122). Indeed, different innovative immunotherapies are in the clinical development for the treatment of patients with RCC. These include inhibitors of ICP, costimulatory agonists, modified cytokines, metabolic modulators, cell therapy and therapeutic vaccines (123).

However, the currently available ICPi are mostly restricted to T cells, since CTLA-4 and PD-1 are expressed on T cells (29). During pregnancy trophoblasts express and secrete members of the B7 family (B7H1/CD274/PD-L1 and B7H3/CD276) as well as HLA-G as immune suppressive proteins (124). Due to the abilities of HLA-G to inhibit various immune effector cell populations including NK cells, CD8$^+$ CTL, CD4$^+$ T helper cells, B cells and other APC, HLA-G appears to be a potent candidate for further anti-tumor immunotherapy aiming on inhibition of immune tolerance/suppression/evasion exerted by HLA-G expressing tumors.

In numerous *in vitro* studies, the downregulation of HLA-G protein levels, e.g. by overexpression of HLA-G regulatory miRs (63), by HLA-G-specific CRISPR/Cas9 systems (125) or by simple inhibition of HLA-G with antibodies (96), resulted in an increased lysis of tumor cells by immune effector cells. Recently, also chimeric antigen receptors (CARs) directed against HLA-G have been published (126).

Beside the advantages of blocking HLA-G alone or in combination with other immunotherapies including antibodies

directed against the checkpoint axes PD-L1/CTLA4 the annual costs per patient receiving ICPi treatment has to be considered. Combination of an anti-HLA-G antibody or antibodies directed against the HLA-G receptors with ICPi might be challenging for financial reasons. The other molecular biological approaches modulating HLA-G expression still requires a long time before successful translation into the clinics, but might offer less expensive alternatives. Another limitation of a possible down-regulation of the HLA-G are putative side effects in regard of the physiological HLA-G expression within the immune privileged tissues. Would a long-term anti-HLA-G therapy increase the risk for irreversible tissue damages by any inflammatory reactions? What are the adverse effects by combining anti-HLA-G therapies with other immunotherapies?

Regarding antibody-based therapies, PD-L1 glycosylation has been shown to lower the binding affinity of respective therapeutic antibodies (avelumab, durvalumab, atezolizumab) (127). It is known that also HLA-G as well ILT2 can be glycosylated (128, 129), but so far no information exists about their glycosylation pattern in RCCs or BCs, which might be associated with a possible negative effect on the affinity of anti-HLA-G antibodies.

WHAT ARE THE FUNCTIONAL CONSEQUENCES OF HLA-G EXPRESSION FOR IMMUNE CELLS?

Soluble HLA-G released by tumor cells interacts with NK cell receptors and CD8$^+$ T cell receptors and even may cause apoptosis of immune effector cells as well as the functional inhibition of immune effector cells. HLA-G suppresses proliferation of CD4$^+$ T lymphocytes (130, 131). In addition, sHLA-G alters CD4$^+$ and CD8$^+$ cells resulting in a loss of their capacity to respond to antigenic stimulation and to their differentiation into immune tolerant Tregs (25). Tregs, DCs and tumor cells can produce and release the anti-inflammatory cytokine interleukin 10 (IL-10), which can promote the expression of HLA-G (29). Membrane-bound HLA-G can affect immune effector cells by trogocytosis, a rapid intercellular transfer of membrane fragments and their associated molecules at intercellular contact (132). In this way, HLA-G can be transferred from tumor cells to activated NK cells or to monocytes. Since transferred HLA-G remains functional, the immune effector cells with acquired HLA-G on their surface do not attack the tumor cells and even gain the capability to inhibit other immune effector cells (29). Beside HLA-G localization on tumor cells, it can also occur in EVs, e.g. in exudates or serum/plasma from cancer patients (133).

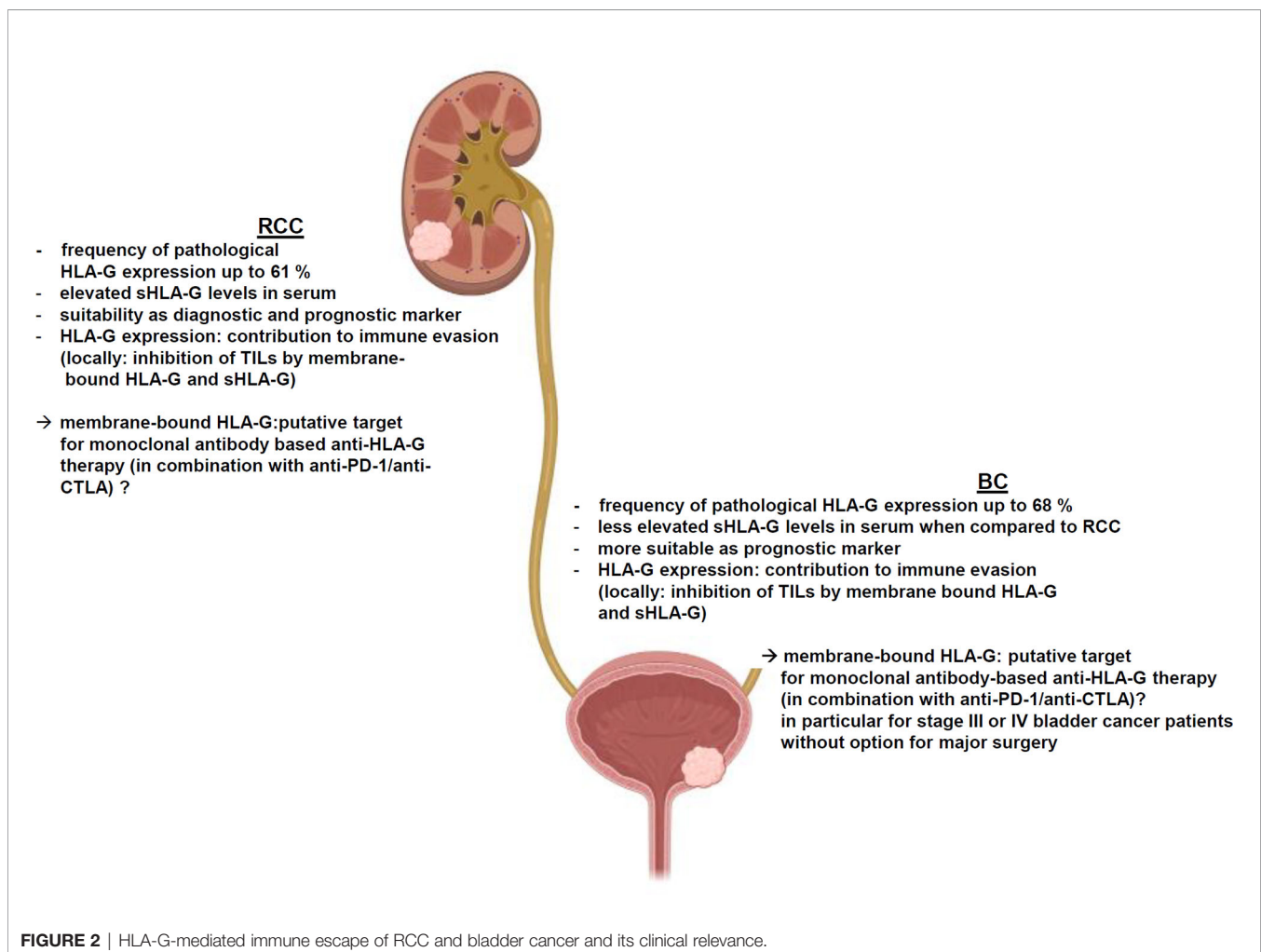## HLA-G AND THE IMMUNE CELL INFILTRATION OF TUMORS

It has been suggested that HLA-G expression can be involved in the immune editing process, which is defined by three distinct

stages of immune responses and the interaction between tumor cells with their microenvironment: the elimination, equilibrium and escape (134). During the elimination phase, HLA-G can inhibit T and B cell activation, proliferation, cytotoxic function of T and NK cells as well as DC function. In the equilibrium phase, HLA-G can downregulate MHC class I expression and induce suppressive myeloid cells (MDSC) as well as regulatory T cells (Tregs) (135–137). The escape phase is characterized by an increased cell proliferative rate and a hypoxic environment. HLA-G has been shown to be induced by the hypoxia inducible factor (HIF)-1 and the vascular epidermal growth factor (VEGF) (94). Furthermore, immune suppressive cytokines, such as IL-10 and TGF-β, are often secreted by tumor cells. For example, in non-small cell lung carcinoma (NSCLC), a loss of classical HLA class I antigens was found to be associated with an upregulation of HLA-G as well as IL-10 expression. In ovarian cancer, HLA-G expression correlated with an elevated expression of tumor marker CA-125 and a combination of both serum markers could improve the clinical screening and diagnosis (138). Multiple studies revealed a broad immune regulatory role of HLA-G affecting innate and adaptive immune responses. Overall, the immune inhibitory mechanisms mediated by HLA-G can be summarized in three main categories: (i) direct inhibition of effector cells and antigen presenting cells, (ii) indirect immune inhibition through induction of regulatory cells and (iii) other mechanisms.

Interestingly, CD8$^+$ ILT2$^+$ T cells in the TME (tumor infiltrating lymphocytes, TIL) show a more mature and aggressive CTL phenotype with a higher cytolytic capacity compared to ILT2-negative peripheral blood precursors or CD8$^+$ PD-1$^+$ TIL. Since HLA-G is able to nearly completely block their activity, this cell pool is an interesting target to release their cytolytic capacity by therapeutic HLA-G inhibition in HLA-G expressing tumors (139). This observation might be particularly interesting for cancer entities, where HLA-G neoexpression has been associated with concomitant high immune infiltration levels, such as Ewing sarcoma and RCC (140).

Indirect immunosuppressive mechanisms are usually based on the induction of durable immune suppressive effects through the generation or induction of Tregs and/or accumulation of myeloid suppressor cells (135). HLA-G is able to impair T helper cell alloproliferation, the regular function of CD4$^+$ T cells and to induce their differentiation into Tregs to indirectly increase

RCC
- frequency of pathological HLA-G expression up to 61 %
- elevated sHLA-G levels in serum
- suitability as diagnostic and prognostic marker
- HLA-G expression: contribution to immune evasion (locally: inhibition of TILs by membrane-bound HLA-G and sHLA-G)

→ membrane-bound HLA-G:putative target for monoclonal antibody based anti-HLA-G therapy (in combination with anti-PD-1/anti-CTLA) ?

BC
- frequency of pathological HLA-G expression up to 68 %
- less elevated sHLA-G levels in serum when compared to RCC
- more suitable as prognostic marker
- HLA-G expression: contribution to immune evasion (locally: inhibition of TILs by membrane bound HLA-G and sHLA-G)

→ membrane-bound HLA-G: putative target for monoclonal antibody-based anti-HLA-G therapy (in combination with anti-PD-1/anti-CTLA)? in particular for stage III or IV bladder cancer patients without option for major surgery

FIGURE 2 | HLA-G-mediated immune escape of RCC and bladder cancer and its clinical relevance.

immunosuppressive effects in the TME (141). Interestingly, HLA-G-induced Tregs are dependent on HLA-G during the differentiation, but not for their immunosuppressive function (142). Transient immunosuppressive effects *via* HLA-G can also be mediated by NK cells, CTLs and monocytes/macrophages, which acquired HLA-G expressing membrane components from other cells *via* trogocytosis (132). Furthermore, HLA-G mediates immune suppression *via* so called "DC-10" dendritic cells (dendritic cells characterized by IL-10 production), which are characterized by high expression of HLA-G and other tolerogenic molecules, such as ILT2 and ILT4 (143). DC-10 cells are known as potent stimulators of allospecific type 1 Tregs, which play a crucial role in promoting and maintaining durable immune tolerance and immune suppression (58, 143). Other immune suppressive mechanisms of HLA-G include the induction of apoptosis of effector cell populations *via* sHLA-G, upregulation of immune inhibitory receptors (including KIR2DL4, ILT2 and ILT4), the suppression of IFN-ɣ release by NK cells (131, 144–147) and inhibition of NK cells and CTL *via* indirect induction of HLA-E expression, which can activate the inhibitory immune checkpoint NKG2A expressed on T and NK cells (148, 149). Furthermore, the HLA-G expression may be induced after infection with human papilloma viruses (150), which are known to have moderate effects on urological malignancies including RCCs and BCs (151).

CONCLUSIONS

HLA-G is frequently, but heterogeneously expressed in both RCC and BC, which is dependent on the tumor subtype, tumor grading or staging as well as the composition of the immune cell infiltration. Unlike other regulatory ICPs, HLA-G exhibits its immune regulatory and immune suppressive functions at multiple levels of the immune response and it is able to either inhibit or stimulate key immune cell populations involved in immune responses to induce potent long-term immune suppression. Despite little information exists regarding the functional link between HLA-G expression and

immune responses, an impaired NK cell- and CTL-mediated recognition of HLA-G-expressing RCC cell lines has been shown, but deserves further investigations in RCC and BC. As summarized in **Figure 2**, HLA-G contributes to the immune escape of both RCC and BC by inhibiting TIL activity due to its high frequency of expression and clinical relevance in both diseases. However, the immune regulatory and immune suppressive functions of HLA-G are considered to be much more profound and complex than those of individual co-inhibitory ICPs, such as PD-L1, CTLA-4 or PD-1, which currently serve as common targets for clinically approved immunotherapies. Recent knowledge offers insights into the underlying molecular mechanisms of HLA-G neoexpression demonstrating a role of HLA-G regulatory miRNAs in RCC. Furthermore, the TME consisting of immune suppressive cytokines secreted by either RCC and BC cells or by different immune cells might impair immune effector responses. In this context, HLA-G might serve as diagnostic and/or prognostic marker or as novel therapeutic target for both malignancies.

Therefore, investigations are urgently required to monitor membranous and sHLA-G in both malignancies in general, but also during immunotherapies of RCC and BC. This will give insights into the potential of HLA-G to serve as target for diverse immunotherapies of HLA-G-expressing tumors than singular inhibition of a less significant ICP – especially in the view of combination therapies.

AUTHOR CONTRIBUTIONS

BS and SJ-B designed the study. BS, SJ-B, ME, HT, SW, CF, RS, and AH wrote the manuscript. All authors contributed to the article and approved the submitted version.

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