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TOPIC HIGHLIGHT

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Inhibition of host immune response in colorectal cancer: Human leukocyte antigen-G and beyond

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Abstract

Colorectal cancer (CRC) is one of the most diffuse cancers worldwide and is still a clinical burden. Increasing evidences associate CRC clinical outcome to immune contexture represented by adaptive immune cells. Their type, density and location are summarized in the Immune Score that has been shown to improve prognostic prediction of CRC patients. The non-classical MHC class I human leukocyte antigen-G (HLA-G), is a crucial tumor-driven immune escape molecule involved in immune tolerance. HLA-G and soluble counterparts are able to exert inhibitory functions by direct interactions with inhibitory receptors present on both innate cells such as natural killer cells, and adaptive immune cells as cytotoxic T and B lymphocytes. HLA-G may play a prominent role in CRC strategies to avoid host immunosurveillance. This review highlights the current knowledge on HLA-G contribution in CRC, in related inflammatory diseases and in other type of cancers and disorders. HLA-G genetic setting (specific haplotypes, genotypes and alleles frequencies) and association with circulating/soluble profiles was highlighted. HLA G prognostic and predictive value in CRC was investigated in order to define a novel prognostic immune biomarker in CRC.

Key words: Colorectal cancer; Human leukocyte antigen-G; Immune score; T lymphocytes; Untranslated regions

Core tip: Colorectal cancer (CRC) prognosis is strictly associated with the immune contexture of tumor microenvironment. IS improves prognostic prediction in CRC. Human leukocyte antigen-G (HLA-G) through its direct inhibitory functions on NK cells and cytotoxic T and B lymphocytes represents a crucial tumor-driven immune escape molecule. This review highlights the current knowledge on HLA-G in CRC and in related inflammatory diseases. HLA-G genetic setting and circulating/soluble profiles need to be defined to comprehend CRC strategies to avoid host immune defences. We suggest that HLA G could represent a novel prognostic immune biomarker to associate with the Immune Score to better characterize host immune response in CRC.

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INTRODUCTION

Colorectal cancer (CRC) remains one of the leading causes of cancer death worldwide[1-3]. CRC develops

sporadically[4], in the setting of hereditary forms[5], or on the basis of inflammatory bowel disease (IBD)[6]. The adenoma-carcinoma transition is the well-established known model for CRC onset[7] and, its genetic and molecular background have been widely described[5,8]. In the last years, the elaborate exchange among innate-adaptive immune cells of the tumor microenvironment, the local inflammatory state, and the host immune response in solid tumors, generated the concept of cancer immunoediting, characterized by the final escape phase exerted by the cancer from host defence immunity[9]. Increasing evidences demonstrated that solid tumors such as CRC are infiltrated by different adaptive cells of the immune contexture that may influence the progression of the disease[10]. These experimental observations finally provided the design of the Immune Score (IS) that is now considered as a novel and independent prognostic marker, in human cancer as well in CRC[11]. IS, is represented by densities of adaptive immune cells: infiltrating $CD8^+$ cytotoxic T lymphocytes (CTLs), and $CD45RO^+$ memory T cells, detected in center and marginal tumor areas[12]. Higher number of infiltrating $CD8^+$ CTLs and $CD45RO^+$ memory T cells correlates with an improved patient prognosis; lower numbers correlate with tumor relapse[13]. IS demonstrated to have a prognostic value for overall survival (OS) and disease free survival (DFS) in retrospective studies[14], and is currently being submitted for clinical validation in prospective CRC studies, in 16 different Countries world wide[15].

The non-classical HLA-G is considered a tolerogenic molecule due to its inhibitory functions *vs* T lymphocytes, NK cells and other cell types of immune contexture[16]. HLA-G is only recently been involved in tumor escape mechanisms from the host immune recognition and destruction[17], and is considered a tumor microenvironment molecule[16,18]. HLA-G shows a lower nucleotide variability in coding sequences, while is highly polymorphic in the untranslated regions (UTRs), both in 5' and 3' segments[19,20] (Figure 1). Polymorphic sites mainly present in the 3'UTR region, may affect the post transcriptional regulation and biological functions of HLA-G[21]. Indeed, through alternative splicing, HLA-G can be expressed as seven different and specific molecules, four membrane bound (HLA-G1 to -G4) and three soluble (HLA-G5 to -G7)[22]. Most of the published data concern the HLA-G1 molecule and its soluble counterpart HLA-G5[23]. HLA-G1 can be shed generating a soluble isoform (sHLA-G1)[17]. The functional characterizations of the five remaining HLA-G isoforms have been yet not clearly elucidated. HLA-G is over-expressed in CRC[24-26] and is a common signature in other types of cancer, autoimmune disorders, viral infections and transplantations[16-18]. Increase in circulating sHLA-G has been detected in CRC in few studies[27,28] and in other malignancies[23,29-31]. Growing attention is focusing on the genetic setting related to the UTRs, especially the 3'UTR involved in micro RNA (miRNA) binding[32]. Single nucleotide polymorphisms (SNPs) in this region have been associated with the disease risk in cancer[33-35] and other disorders[36,37] (Table 1), but the association with CRC and the prognostic value in this type of malignancy, remain to define. Aim of this review was to investigate the HLA-G as a potential prognostic biomarker in CRC and related inflammatory colon diseases, both at the genetic and circulating profiles (Table 2). Genotypic-phenotypic correlation has been highlighted and its potential role in IS explored (Figure 2).

CRC AND CHRONIC COLONIC INFLAMMATION

CRC is one of the most diffuse cancers worldwide with about 1.2 million new cases and 600000 deaths recorded annually[1].

Despite improvements and advances in diagnosis, surgery and treatment, CRC is still the 2nd most common cause of cancer death in the United States and other industrialized countries[2,3].

Development of sporadic (88%-94%) and hereditary forms of CRC are mainly related to the accumulation of genetic changes in gatekeeper and caretaker genes like *APC* and other oncoproteins (*K-ras*, *erb-s*, *c-src*, *β-catenin, PI3K*) and tumor suppressors (*p53*, *Smad4*)[4,5], according to the aberrant crypt foci (ACF)-adenoma-carcinoma transition model[7]. It is well-established that the acquisition of these mutations in a multistep mechanism is part of the genomic instability process that comprehends the chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP) pathways[8]. While CIMP exhibits gene silencing due to hypermethylation of CpG islands, in CIN positive tumors (about 70% of sporadic CRCs) an imbalance in chromosome number (aneuploidy), subchromosomal genomic amplifications and a high frequency of loss of heterozygosity (LOH) are observed[38]. About 15% of sporadic CRCs, mostly in the proximal colon anatomic site, are characterized by MSI with a large number of mutations at microsatellite sequences interesting the DNA mismatch repair (MMR) system, so the consequence is the accumulation of thousands of unrepaired mutations[39,40].

Genesis of most of CRCs depends also from environmental factors like intestinal microbiota, dietary habits and lifestyle, associated with the patient genetic background[41]. The chronic colonic inflammation due to active ulcerative colitis (UC) or Crohn's disease (CD), the two major forms of inflammatory bowel disease (IBD), lead to the colitis-associated cancer (CAC) development that is a CRC subtype[6]. In UC, inflammation is limited to the mucosal layer and usually starts from the rectum spreading then into the colon, while in CD all the layers of gut wall are interested with the terminal ileum and also the colon as the most common sites[42].

IBD subjects are at increased risk to develop the tumor; it is estimated that about 20% of patients affected by chronic UD and CD for a long time, within 30 years, develop CAC; thus CRC risk increases with the duration

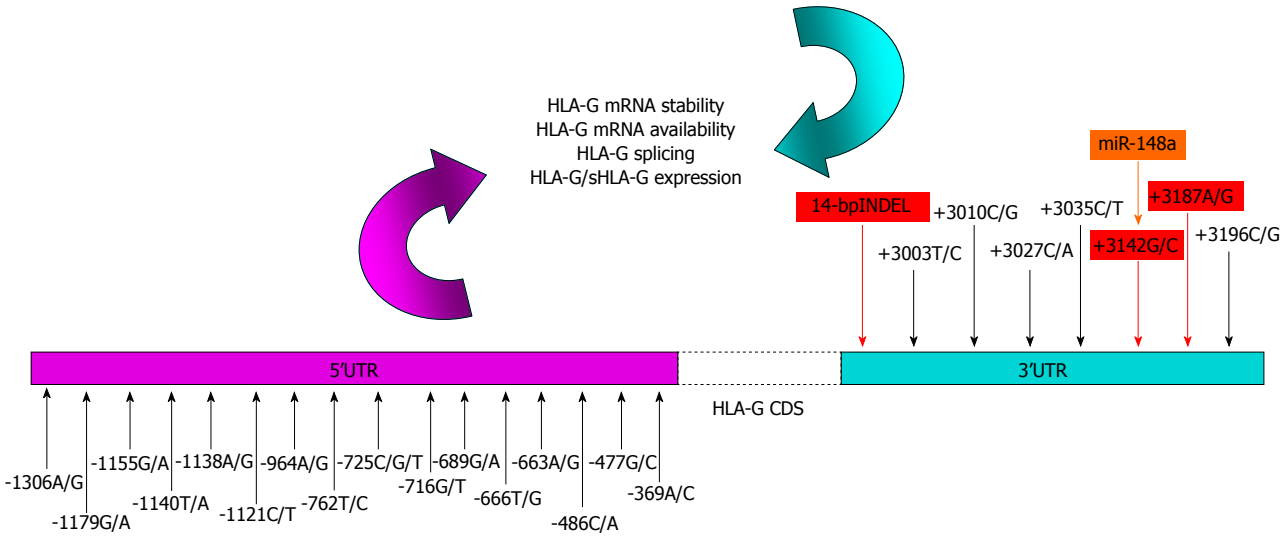

Figure 1 Human leukocyte antigen-G single nucleotide polymorphisms involved in the biological features of the protein: nucleotide variants in the 5'-3' untranslated regions may influence human leukocyte antigen-G expression levels by modifying the affinity of gene targeted sequences for transcriptional (5') or post-transcriptional (3') factors respectively. Polymorphisms in 5'UTR (fucsia) were previously described by Costa *et al*[113] those in 3'UTR (light blue) by Castelli *et al*[19]. In red 3'UTR SNPs involved in HLA-G mRNA stability and availability are highlighted. In orange the only one microRNA with a demostrated functional inhibitory role in the *HLA-G* expression[134] was highlighted . UTR: Untranslated region; SNPs: Single nucleotide polymorphisms; HLA-G: Human leukocyte antigen-G.

Table 1 List of associations found between single nucleotide polymorphisms/alleles in human leukocyte antigen-G untranslated regions and different pathologies; genotype-phenotype correlations were also reported

UTRs	SNP	Genotype and/or allele	Disease	Association with[1]	UTR SNP and sHLA-G correlation	sHLA-G level	Statistical significance[2]	Country	Ref.
3′	14 bp INDEL	14 bp I/I	PE	Increased disease risk	ND	ND	Yes	China	[36]
3′	14 bp INDEL	14 bp I/I	RSA[3]	Increased disease risk	ND	ND	No	Denmark	[112]
3′	+3142 C/G	+3142 GG and G allele	SLE	Increased disease risk	ND	ND	Yes	Brazil	[37]
3′	14 bp INDEL	14 bp I/I	SLE	Increased disease risk	ND	ND	No	Brazil	[37]
3′	14 bp INDEL	14 bp I/I	OvaC	Increased disease risk	ND	ND	Yes	Canada	[33]
3′	14 bp INDEL	14 bp D/D	EsophC	Increased disease risk	ND	ND	Yes	China	[34]
3′	14 bp INDEL	14 bp D/D and D allele	HCC	Increased disease risk	ND	ND	Yes	Brazil	[35]
3′	14 bp INDEL	14 bp I/I and D allele	HCC	Increased disease risk	ND	ND	No	South Korea	[137]
3′	14 bp INDEL	14 bp I/I	Allo-HSCT	Lower OS and DFS	ND	ND	Yes	Italy	[138]
3′	14 bp INDEL	14 bp D/D	RA	MTX therapy (responder group)	Yes	Higher	Yes[4]	Italy	[114]
3′	14 bp INDEL	14 bp I/I	RR-MS	sHLA-G	Yes	Lower	Yes[4]	Italy	[139]
3′	+3142 C/G	+3142 GG	RR-MS	sHLA-G	Yes	Lower	Yes[4]	Italy	[139]
3′	14 bp INDEL	14 bp I/I	IVF[3]	sHLA-G	Yes	Absent	Yes	Denmark	[141]
5′	-725C/G/T	-725C>G	IVF[3]	sHLA-G	Yes	Absent	ND	Denmark	[141]
3′	14 bp INDEL	14 bp I/I	Heart T	sHLA-G	Yes	Lower	Yes	Canada	[142]
3′	14 bp INDEL	14 bp I/I	HD	sHLA-G	Yes	Lower	Yes	China	[143]
3′	14 bp INDEL	14 bp D allele	ERA	Improved disease remission	Yes	Higher	Yes[4]	Italy	[144]
5′	-725C/G/T	-725Callele	RPL[3]	sHLA-G	Yes	Lower	Yes	Iraq	[148]
3′	14 bp INDEL	14 bp I/I	PTC	Increased disease risk not found	No	Higher	Yes[5]	Italy	[31]

[1]Data reported in this column are related to the association with SNPs in UTRs; the increased disease risk was compared with a HD control group; [2]Statistical significance is referred to HLA-G SNP and disease status analysis; [3]The disease status is related to infertility problems; [4]A statistical significance was found also between HLA-G SNP and sHLA-G levels; [5]A statistical significance was found between sHLA-G levels and the disease risk. UTR: Untranslated region; SNP: Single nucleotide polymorphism; sHLA-G: Soluble human leukocyte antigen-G; INDEL: Insertion/deletion polymorphism; I: Insertion; D: Deletion; PE: Pre-eclampsia; RSA: Recurrent spontaneous abortions; SLE: Systemic lupus erythematous; OvaC: Ovarian cancer; EsophC: Esophageal cancer; HCC: Hepatocellular carcinoma; Allo-HSCT: Allogeneic hematopoietic stem cell transplantation; RA: Rheumatoid arthritis; RR-MS: Relapsing-remitting multiple sclerosis; IVF: *In vitro* fertilization failure; Heart T: Heart transplantation; HD: Healthy blood donors; ERA: Early rheumatoid arthritis; RPL: Recurrent pregnancy loss; PTC: Papillary thyroid carcinoma; OS: Overall survival; DFS: Disease free survival; MTX: Methotrexate; ND: Not determined.

of the IBD and the severity of inflammation[43].

Pathogenesis of CAC in chronic colitis patients differs from the classical model sustained by sporadic CRC, for a transition from low-high grade dysplasia to carcinoma and also for the sequence of cellular and molecular events[44]. In CRC adenomatous polyposis coli (APC)

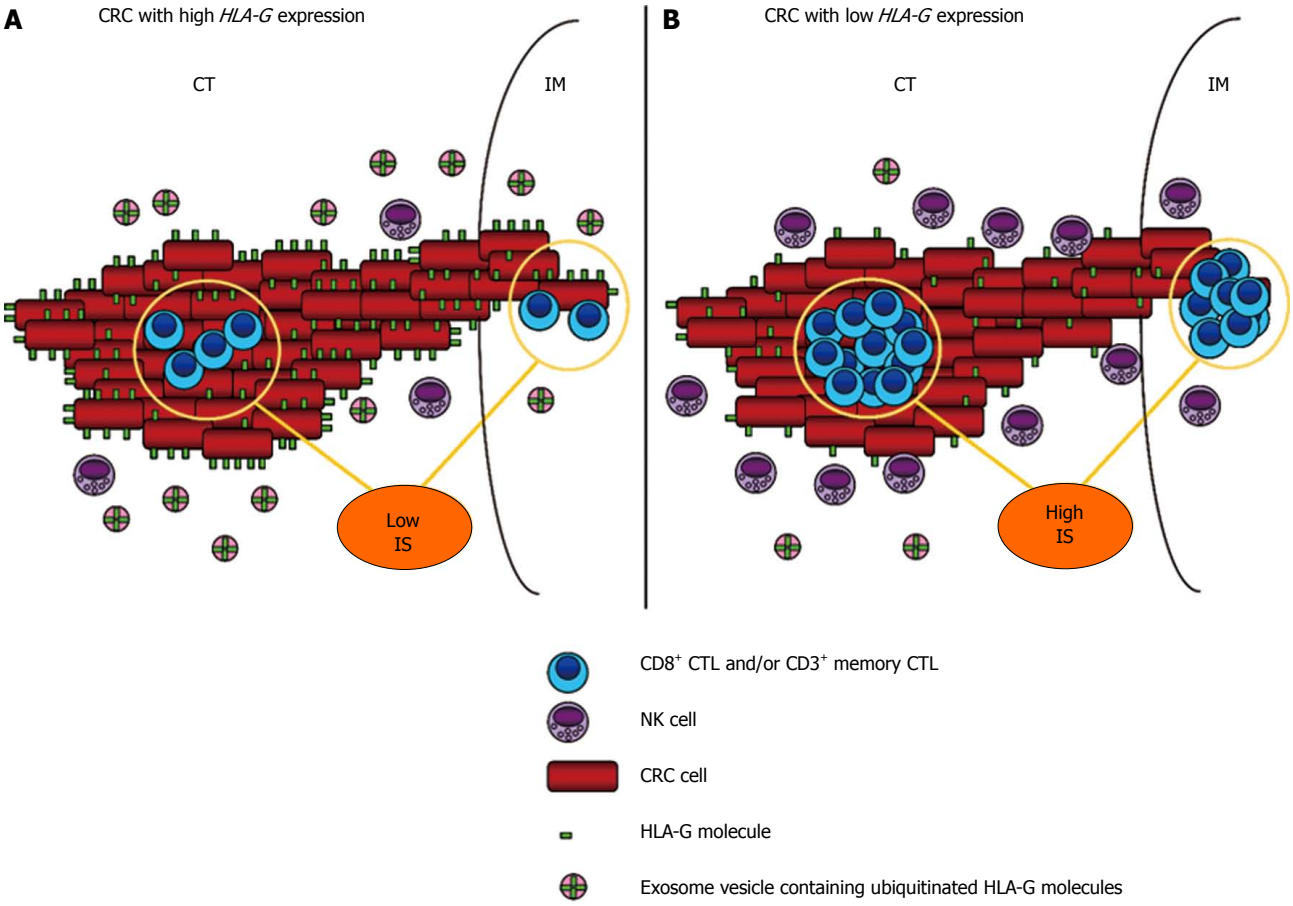

Figure 2 Schematic representation of colorectal cancer tumor microenvironment: potential influence of human leukocyte antigen-G expression on Immune Score and host immune local response. A: High *HLA-G* expression shows an inhibitory role against host immune defences, represented mostly by natural killer cells and CTLs. Increased *HLA-G* expression in the local microenvironment is also supported by exosome contribution through a hypothetical trogocytosis release mechanism that mediates HLA-G up-take. Another consequence is a direct association with a low IS value due to HLA-G inhibitory functions on CTLs in CT/IM areas of the tumor, and consequently with a worse patient prognosis (not represented); B: Lower HLA-G expression correlates with a high IS value and a favoureable immune contexture that improves host immune response and patient prognosis (not represented). Representation of IS is referred to recent quality and validation criteria[15] (see text for further clarifications). HLA-G: Human leukocyte antigen-G; CTLs: Cytotoxic T lymphocytes; IS: Immune Score; CT: Center of the tumor; IM: Invasive margin.

protein loss of function, is an early event during the formation of precocious adenoma, while it is less frequent and occurs in the late pathogenesis of CAC[45]. p53-loss of heterozygosity (LOH), *p53* mutations or loss of function are early molecular events characterizing CAC origin instead of CRC in which they frequently occur in the late adenoma-carcinoma transition[46,47].

IMMUNE CONTEXTURE IN INFLAMMATION AND CRC

The chronic inflammatory status is a common feature of colorectal and colitis-associated tumors. Chronic inflammation is modulated by immune innate/adaptive cells infiltration and immune microenvironment[48,49]. The crucial role of inflammation in tumorigenesis is emerged only recently and now it is estimated that about 15%-20% of cancers are related to an underlying chronic inflammation process[50,51]. CAC is considered a classical inflammation-driven cancer as demonstrated in mice models in presence of dextran sodium sulphate (DSS) and the procarcinogen azoxymethane (AOM)[52].

A growing body of evidence is focusing both on relationships between the immune contexture of solid tumors like CRC, and patient prognosis in terms of DFS and OS[53]. Tumor microenvironment is represented by a complex network of stromal, inflammatory and immunocompetent cells. Histopathological analyses show that solid cancers are infiltrated by innate-adaptive immune cells, that have a role in tumor growth[54,55].

The interplay among tumor cells and immune system is highly complex and suggested the concept of cancer immunoediting, a dual process in which the main actors are the host-protective and the tumor-promoting actions of immunity[9,56,57]. Cancer immunoediting occurs in three phases: elimination, equilibrium and escape. In the elimination phase (the modern concept of the older notion "cancer immunosurveillance")[58], innate and adaptive immunity work together to recognise and destroy nascent tumor cells. If tumor cell variants are not completely eliminated, will enter into an equilibrium phase, in which adaptive immunity controls and stems the growth of

Table 2 Summary of human leukocyte antigen-G evaluations in colorectal cancer and related colonic diseases of the gastrointestinal tract

Sample type	HLA-Gs	Methods	Disease, *n*	Relevances	Ref.
Tumor DNA	HLA-G	RT-PCR	CRC, *n* = 39	HLA-G mRNA was significantly more expressed in CRC (87.2%) than in the extra neoplastic tissue	[24]
Tumor tissue	HLA-G	IHC	CRC, *n* = 201	HLA-G is over-expressed in primary CRC sites (64.6%), but not in the normal CRC tissues or benign adenomas	[25]
Tumor tissue	HLA-G	IHC	UC, *n* = 24; CD, *n* = 19	HLA-G and IL-10 are highly expressed in UC but not in CD tissue biopsies	[154]
Tumor tissue	HLA-G	IHC	CRC, *n* = 60; DA, *n* = 67; BC, *n* = 37; AC, *n* = 52	HLA-G is over-expressed in 52 % of CRC lesions and also in 79% of PDAs, 76% in BC and 75% AC	[26]
Tumor tissue	HLA-G	IHC	CRC, *n* = 415	HLA-G is expressed in > 30% of CRC lesions (data summarize published data collected until 2008)	[16]
Tumor tissue	HLA-G	IHC	CRC, *n* = 154	HLA-G is expressed in > 30% of CRC lesions (data summarize published data collected until 2005)	[17]
Serum	sHLA-G	ELISA	CRC, *n* = 144	Higher sHLA-G levels in CRC (median 124.3 U/mL) compared to benign colorectal diseases (cut off value 88.6 U/mL). CEA showed less sensitivity e specificity	[27]
Plasma	sHLA-G	ELISA	CRC, *n* = 37	sHLA-G as a diagnostic biomarker for the detection of early CRC (median 84 U/mL) with respect to BD (median 34 U/mL)	[28]
PBMC	sHLA-G	ELISA	HD, *n* = 30; CD, *n* = 10; UC, *n* = 18	Spontaneous secretion of sHLA-G from cultured PBMCs of CD but not in UC and BD Secretion of sHLA-G in CD patient cultures and BD but no in UC, after LPS stimulation	[161]
Plasma and PBMC	HLA-G	ELISA	UC, *n* = 27; CD, *n* = 22	Immunosuppressive therapy decreases sHLA-G hyperproduction in CD and induces its release in UD, in both plasma and in PBMC culture supernatants	[162]

HLA-G: Human leukocyte antigen-G; sHLA-G: Soluble HLA-G; CRC: Colorectal cancer; UC: Ulcerative colitis; CD: Crohn's disease; PDA: Pancreatic ductal adenocarcinoma; BC: Biliary cancer; AC: Ampullary cancer; HD: Healthy blood donors; PBMC: Peripheral blood mononuclear cells; IHC: Immunohistochemistry; LPS: Lipopolysaccharide; CEA: Carcinoembryonic antigen; IL: Interleukin; ELISA: Enzyme-linked immunosorbent assay.

clinically undetectable tumor cells and blocks tumor cell immunogenicity. When dormancy of tumoral cells stops, malignant cells with reduced immunogenicity shift into the escape phase and begin to grow and proliferate in an immunologically unrestrained way, establishing an immunosuppressive tumor microenvironment, and becoming clinically detectable. Escape mechanism from immune host control is now considered one of the hallmarks of cancer[59]. Innate immunity is the first line of host defense and it is specialized in counteracting cancer cells and virally infected cells.

Innate immune cells *i.e.*, myeloid derived suppressor cells (MDSCs), macrophages, neutrophils, dendritic cells (DCs), mast cells (MCs), and NK cells, may have a pro- or anti-tumorigenic role in both CRC and CAC[60]. MDSCs under the control of nuclear factor κB (NF-κB), produce interleukin (IL)-6 that activates transcription factor signal transducer and activator of transcription 3 (STAT3) resulting in survival, growth and progression signals in early CAC[61].

Macrophages are responsible mainly for pro-inflammatory cytokines release and are distinguished in two types. Type 1 macrophages (M1) that are activated by interferon (IFN)-γ or tumor necrosis factor (TNF) cytokines, are efficient producers of reactive oxygen and nitrogen species, have an interleukin (IL)-10^{low} IL-12^{high} IL-23^{high} inflammatory phenotype, and tend to negatively control tumor growth[62]. Conversely, type 2 macrophages (M2) share an IL-10^{high} IL-12^{low} IL-23^{low} anti-inflammatory phenotype and stimulate cancer proliferation by secreting immunosuppressive cytokines like IL-10. Production of mediators promoting angiogenesis such as Vascular Endothelial Growth Factor A (VEGFA) and Cyclo-Oxygenase-2 (COX-2)-derived prostaglandin E2[63], hypoxia-dependent upregulation of chemokines (C-X-C motif), induces accumulation of M2 macrophages that is the predominant phenotype in tumor microenvironment[64,65]. A macrophage polarization of tumor-associated macrophages (TAMs) due to the local colonic microenvironment during tumor progression is observed with a switching from M1 (inflammatory) to M2 (anti-inflammatory) type and a gradual NF-κB inhibition[66]. TAMs are one of the major components of leukocyte infiltrates in tumors and represent an independent prognostic factor of poor prognosis in multivariate analysis in different malignancies[67]. In CRC, TAMs correlate with improved OS[68]. Finally, innate immune cells orchestrate a complex inflammatory environment that may be modulated to either stimulate or inhibit CRC proliferation[69,70].

While innate immunity does not involve a specific antigen or peptide or particular tumor-associated antigen recognition, this is a prerequisite for adaptive immunity. Cells of adaptive immune system are mainly represented by B lymphocytes, T helper 1 (Th1) and T helper 2 (Th2) CD4$^+$ cells, CD8$^+$ Cytotoxic T lymphocytes (CTL) cells and CD4$^+$ T regulatory (Treg) cells[71]. Recent findings of memory responses by NK cells suggest that also NK may contribute to adaptive immunity[72]. To destroy cancer cells, CTLs need to recognize an antigen exposed on the tumor cells in association with the human leukocyte antigen (HLA) class I proteins[73]. Only through recognition of this tumor cell antigen/HLA I complex for which

their T cell receptor (TCR) is specific, CTLs clonally expand and differentiate in memory T cells[74] ($CD45RO^+$). $CD45RO^+$ comprise $CD3^+$, $CD4^+$ and $CD8^+$ T cells that have been exposed to antigen and respond faster and with an increased intensity after antigen stimulation compared with naïve T cells[54].

Upon activation, CTLs release proteases and lytic components as perforin and mediate disruption of tumor cell membrane and activation of apoptotic pathway. $CD4^+$ T cells respond only to antigens presented by the HLA class Ⅱ proteins expressed by DCs in secondary or tertiary organs[75]. Many evidences highlight that one of the cancer immunoediting topic is due to the fact that T-cell recognition of tumor antigens drives the immunological destruction of nascent and developing cancer cells. One of the most well-known way used by tumors to escape from specific T-cell recognition mechanisms is down-regulation or total suppression of MHC I molecules, and specific alteration leading to the inefficient presentation of immunodominant antigens[76].

Recently, Matsushita *et al*[77], demonstrated that the immunoselection by $CD8^+$ T cells of tumor variants lacking strong tumor-specific antigens represents one of the mechanism by which cancer cells escape tumor immunity.

Th1 cells secrete cytokines like IFN-γ and TNF-α, support tissue destruction and CTLs by producing IL-2 required for $CD8^+$ proliferation[78]. Th2 cells produce cytokines such as IL-10, IL-4 and IL-5, and limit CTLs proliferation. Tregs that also highly express CD25 ($CD4^+CD25^+$) secrete IL-10 and Tumor Growth Factor (TGF)-β which dampen the immune response[79]. It should be emphasized that while CTLs, Th1 and Th17 inhibit cancer growth, Th2 cells and Tregs stimulate cancer proliferation. Moreover, a shift in Th1 (tumor rejection)/Th2 (tumor promotion) immune response is observed in CRC[80]. Restoring of normal immunological functions and pro-inflammatory cytokines after tumor resection in CRC were demonstrated, highlighting that CRC itself has a direct immunosuppressive effect[81,82].

Overall, immune contexture should be considered as comprising the density of $CD8^+$ CTLs and $CD45RO^+$ memory T cells, their location at the center of the tumor (CT) and invasive margin (IM), combined with the quality of tertiary lymphoid structures (TLS) and additional functionality entities such as Th1-related factors, chemokines, adhesion molecules and cytotoxic factors[83]. Indeed, in CRC, not only Th1 immunity markers (STAT1, IRF1, IFN-γ-SG pathway), cytotoxic markers (Granzyme Perforin, Granulysin, TIA1, Caspase pathway), but also chemoattraction (specific chemokines such as CX3CL1, CXCL10, CXCL9) and adhesion (molecules as ICAM1, VCAM1, MADCAM1) signatures are relevant in influencing the density of infiltrating immune cells[84,85].

CRC AND IMMUNE SCORE

Accumulating evidences since the late 1990s showed an association among tumor-infiltrating lymphocytes (TILs) able to inhibit cancer growth, and improved prognosis in CRC[10,86] and other malignancies such as melanoma[87] and ovarian cancer[88]. A particular phenotype in CRC is represented by MSI type tumors that are associated with a high TILs levels, loss or downregulation of HLA class Ⅰ, better patient prognosis, a reduced metastatic potential, and a different response to chemotherapy[39,60].

In 2005-2006 the idea that the adaptive immune response plays a role in preventing tumor recurrence in CRC emerged, mostly from the works of Pagès *et al*[89] and Galon *et al*[13]. The authors first demonstrated that CRCs with high density of infiltrating and effector memory T cells with a protective immune role, were less prompt to metastasize and can be associated with an increased survival of patients[89]. Subsequently, using the same cohort of patients, relationships among type, density and location of immune cells within the tumor and the clinical outcome, were investigated by using both genomic approaches and immunohistochemistry (IHC)[10]. Through the selection and the evaluation of expression levels of genes involved in inflammation, Th1 adaptive immunity and immunosuppression, Galon *et al*[13] found a dominant cluster of co-modulating genes for Th1 adaptive immune response (*i.e.*, IFNG, CD8a, GLNY, GZMB, CD3z). Applying specific tissue microarrays and a dedicated image analysis work station, a quantification of total ($CD3^+$) T lymphocytes, $CD8^+$ CTL effectors, associated molecule (GZMB), and $CD45RO^+$ memory T cells, was performed both in CT and IM.

High immune cell densities ($CD3^+$, $CD8^+$, GZMB and $CD45RO^+$) in both CT and IM tumor regions were present in CRC patients without recurrence after adjuvant therapy, while lower densities of the same immune cell types correlated with disease recrudescence. Results highlighted an inverse correlation among expression of these genes and CRC relapse suggesting that Th1 adaptive immunity improve clinical outcome[13]. Camus *et al*[14] demonstrated the association between loss of coordinated functional immune reaction and the progression of CRC to a metastatic phenotype. These preliminary results demonstrated for the first time that the host immune response plays an important role in determining the outcome of CRC patients. Type, density, and location of immune cells in CRCs increase the prediction accuracy of DFS and OS, and started to represent a superior and independent prognostic parameter with respect to the UICC-TNM well accepted classification[90].

Finally, all these data and evidences, culminated in the concept of "Immune Score" that emerged for the first time in 2011 in the work of Pagès *et al*[12]. A multivariate Cox proportional hazard regression model was used to assess the hazard ratio of the immune score combination (CD45RO/CD8) in specific tumor regions (CT/IM), together with clinical and histopathological tumor markers. Pagès *et al*[12], analyzing a large cohort of CRC patients with early (Ⅰ-Ⅱ) stage, showed that the combined analysis of cytotoxic ($CD8^+$) and memory ($CD45RO^+$) T cells confirmed its prognostic discrimina-

tory power in the prediction of tumor recurrence and survival[12]. Subsequently, the concept of IS as a clinical prognostic marker improving the standard TNM classification at any stage of CRC, has been established evaluating infiltrating lymphocytes of 599 CRC specimens by Mlecnik *et al*[91]. Patients with high IS had increased DFS and OS, and patients with a low IS were likely to experience a disease relapse[92]. IS represents a standardized, simple and powerful immune stratification system proposed as a novel prognostic immune marker for routine testing potentially helpful for CRC management to better identify and stratify high-risk patients who would benefit most from adjuvant therapy[12].

Cancer outcomes can vary significantly among patients with the same stage and this could be related to differences in immune cells densities from patient to patient as in CRC[93]. This argues the limit of traditional AJCC/UICC TNM classification in providing limited prognostic information and predicting response to therapy[94]. A worldwide task force representing 22 Institutions from 16 different countries, is working now for IS validation in clinical practise, with the aim to introduce it as a new component of classical cancer classification, that will maybe designate in future as TNM-I (TNM-Immune)[15]. To improve quality and validation in standard laboratories, IS will be quantified by the combination of the two easiest membrane stains CD3 and CD8 to avoid any background noise due to CD45RO and GZMB stains[15]. Special emphasis will be focused in the prognostic significance of validated immunologic parameters in CRC that with the primary goal to validate the prognostic power of the IS in routine settings of stage Ⅰ/Ⅱ/Ⅲ CRC patients and for recurrence prediction for stage Ⅱ CRC patients[95]. Thus, the bases to revise and renegotiate the clinical outcome based on classical clinical parameters have been seeded[96].

Recently, a great emphasis has been done in an attempt to define the prognostic role of another subtype of tumor infiltrating cells. Treg cells also positive for the nuclear transcription factor protein forkhead box P3 (Foxp3), showed a strong and independent prognostic significance in CRC, superior to $CD45RO^+$ and $CD8^+$ cells[97]. $Foxp3^+$ Tregs cells are generally associated with immunosuppressive properties and poor prognosis in different solid tumors such as hepatocellular[98], prostate[99] and pancreatic carcinoma[100], but conversely were reported to be associated with improved prognosis in CRC[97]. Although Foxp3 is a well accepted marker used to identify tumor infiltrating $CD4^+$ $CD25^+$ Tregs, it is known that a small proportion of $Foxp3^+$ cells may also be $CD8^+$. $CD8^+$ $CD25^+$ $Foxp3^+$ T cells showed suppressive capacities in CRC[101], suggesting that $Foxp3^+$ role should be more explored. Foxp3 expression evaluated in tumor cells was associated with worse outcome of patients in different solid tumors[102-104] but not in CRC, highlighting a new independent prognostic factor. Recently, $Foxp3^+$ expression in tumor cells was compared to $Foxp3^+$ Treg infiltration in CRC, demonstrating for the first time an inverse correlation between the number of $Foxp3^+$ Treg and the level of $Foxp3^+$ in tumor cells, suggesting an anti-proliferative effect of TGF-β on Tregs[105]. Furthermore, patients with high $Foxp3^+$ expression profile in CRC tumor cells were correlated with a poorer prognosis that was not observed for $Foxp3^+$ Treg in the tumor[106].

HLA-G: A CRUCIAL TUMOR-DRIVEN IMMUNE ESCAPE MOLECULE

HLA-G is considered as a tolerogenic molecule exerting its inhibitory functions by direct interaction with different inhibitory receptors of the immunoglobulin family present on NK cells (ILT2/CD85j, KIR2DL4/CD158d), T lymphocytes (ILT2/CD85j, KIR2DL4/CD158d), B cells (ILT2/CD85j), endothelial (CD160), macrophages, monocytes and DCs (ILT2/CD85j, ITL4/CD85d)[16]. HLA-G expression was originally detected in non-pathologic conditions and restricted to extravillous cytotrophoblast, thymic epithelial cells, cornea, pancreas, erythroid and endothelial precursor cells[18]. Recent studies showed that HLA-G proteins can be detected also in pathological conditions, such as in allografts and infiltrating immune cells within transplanted tissues, inflammatory diseases, virus infections and cancer[107-109]. Originally, durings the 1990s, HLA-G role in maternal-fetal tolerance preventing attack of the fetus by the maternal immune system by its interaction with uterine NK cell inhibitory receptors was demonstrated[110]. This binding through KIRDL4 receptor stimulates secretion of cytokines and angiogenic factors from NK cells, which favours implantation and placental vascularisation and development. Trophoblast itself secretes HLA-G modulating balance among these proangiogenic and antiangiogenic factors. The tolerogenic role of HLA-G in pregnancy is strongly supported by the correlations between HLA-G down regulation and preeclampsia/spontaneous abortions events[111-113]. These preliminary evidences observed in maternal-fetal tolerance suggested also that microenvironment factors may modulate HLA-G expression in tissues. Ectopic expression of HLA-G in damaged cells or tissues may be enhanced by stress, nutrient deprivation, hypoxia, hormones such as progesterone, cytokines (GM-CSF, IFNs, IL-10, TNF-α, TGF-β, LIF)[16] and immunosuppressive drugs[114].

HLA-G gene is composed of eight exons and seven introns with a stop codon at exon 6, a quite large 5'UTR extending at least 1.4 kb from ATG, and a 3'UTR[32]. The coding exons transduce only the heavy chain of the molecule and are located on chromosome 6, while β2-microglobulin (β2m) is encoded by a separated gene on chromosome 15. Exon 1 encodes the signal peptide, exons 2, 3 and 4 the extracellular α1, α2 and α3 domains respectively, and exons 5 and 6 the transmembrane and the cytoplasmic domain of the heavy chain. Exon 7 and 8 are not translated. The 3'UTR is included in the exon 8[21]. Classical HLA class I molecules are characterized by nucleotide sequence variations around the peptide-

binding cluster encoded by exon 2 (α1 domain) and exon 3 (α2 domain), while HLA-G nucleotide variability spans through exon 2, 3 and 4 (α3 domain)[21,22]. HLA-G coding sequence has a limited genetic variability in contrast to the classical HLA class I molecules that exhibit hundreds of alleles and, to date, 49 different alleles and 15 related proteins have been recognized[115]. On the other hands, 5' UTR and 3'UTR segments are high polymorphic, both influencing *HLA-G* expression modifying the affinity of gene targeted sequences for transcriptional or post-transcriptional factors, respectively[19] (Figure 1).

Indeed, HLA-G may presents 7 protein isoforms generated by alternative splicing of the primary transcript. 4 isoforms are membrane-bound (HLA-G1, G2, G3 and G4) and 3 are soluble (G5, G6 and G7) species[23]. HLA-G1 and HLA-G5 are the most common isoforms observed, but HLA-G1 was the first isoform to be discovered in healthy tissues and first implicated in maternofetal tolerance[116]. HLA-G1 is the complete protein similar to the other classical membrane bound HLA-I molecules associated with β2m[23]. Crystal structure of the protein have shown that the full-length HLA-G1 is composed of a heavy chain non-covalently associated with the β2m molecule, and a peptide of about 8-10 amino acids similar to that found in the other classical I HLAs[117]. HLA-G2 isoform has no α2 domain, HLA-G3 has no both α2 and α3 domains, HLA-G4 does not present α3 domain[21]. This high post transduction availability in HLA-G molecules suggests a deeper modulation due to alternative splicing involving mostly 3'UTR region. We speculate that it could be tissue specific considering that miRNA are differentially expressed, and this modulation may be influenced by inflammation status and immune contexture microenvironment. It is known that KIR2DL4 present on NK and T cells binds HLA-G through α1 domain, or ITL-4 (DCs, monocytes and macrophages) and CD8 (T and NK cells) bind *via* α3 domain, or ITL-2 *via* α3 domain in association with β2m, however, the exact role of the less common HLA-G isoforms has not been elucidated[118]. HLA-G dimers may also be formed *via* a Cys42-Cys42 intermolecular disulfide bond on the α1 domains of the heavy chains from two HLA-G monomers. These HLA-G dimers exhibit enhanced binding avidity for ITL2/4-mediated signaling[119]. Soluble and not bound HLA-G5, HLA-G6 and HLA-G7 species have the same extraglobular domains of HLA-G1, HLA-G2 and HLA-G3 respectively[21]. It should be pointed that another form of HLA-G may be generated by proteolytic shedding of the isoform HLA-G1 (sHLA-G1)[120] and potentially anchored HLA-G2-G4, can also be shed from the cell surface if expressed. It should be highlighted that sHLA-G1 is analogue to the sHLA-G5 isoform. Soluble isoforms consequent to secretion or shedding, especially the most common sHLAG5 and sHLA-G1, can be detected in body fluids such as plasma, serum, ascites, cerebro spinal fluids exudates from patients with inflammatory diseases o cancer[17,121,122]. Similarly to membrane bound HLA-G1, sHLA-G exerts immunosuppressive functions *vs* cells of the immune contexture. Furthermore, sHLA-G produced by immune cells and/or by cancer cells induces apoptosis of activated $CD8^+$ T cells by binding to CD8 and by triggering a Fas/FasL-dependent pathway[119]. Moreover, the release of IL-3, IL-4 and IL-10 is stimulated by sHLA-G[17]. Increased sHLA-G levels compared to healthy controls have been reported in successful pregnancy after *in vitro* fertilization (IVF)[121], in patients with lymphoproliferative disorders[29], melanoma[30], and different type of cancers[23,31]. sHLA-G has been proposed as a diagnostic biomarker in CRC with increased specificity and sensibility respect to the well known carcinoembryonic antigen (CEA) protein[27]. Due to the possibility that serum sHLA-G is trapped within during the clot formation, to evaluate true biological sHLA-G levels, it is recommended to detect sHLAG in plasma[123]. Anyway, many studies still present data from serum samples.

sHLA-G levels are commonly detected with classical ELISA assay by the use of the monoclonal antibody (MoAb) MEM-G9 which recognizes (the precise epitope is unknown) the native HLA-G molecule in β2m associated forms, HLA-G1 and HLA-G5 soluble and not membrane bound isoforms[124]. It should be noted that recently, Zhao *et al*[125] demonstrated by flow cytometry that the MEM-G9 antibody it is able to bind also HLA-G3 isoform that is β2m free, thus speculating that an epitope on MEM-G/9 localized on the α1 domain of HLA-G. HLA-G may be expressed at the cell surface and also secreted. Anyway, sHLA-G can also be produced into the cells and subsequently incorporated in microvesicles, the exosomes such as in cancer[126] (Figure 2). In exosomes, HLA-G molecules form high molecular weight complexes through disulfide bridges, share partially an ubiquitinated phenotype and can be released by exosomes as demonstrated recently *in vivo*[127]. Moreover, very preliminary data coming from the HLA-G Conference held in Paris in 2012, showed that in plasma samples of healthy controls sHLA-G is preferentially exosomal-bound, while in plasma samples of lung cancer patients the level of free "not exosomal-bound" sHLA-G is increased[128]. Intriguing, it was demonstrated a mechanism of protection and immune evasion for HLA-G negative tumor cells that are in proximity of HLA-G positive tumor cells. These HLA-G expressing tumor cells by "trogocytosis" transfer membrane patches containing HLA-G molecules to active and surrounding NK cells[129]. Upon acquisition of HLA-G1-containing membranes from tumor cells, effector NK cells stop proliferating, stop being cytotoxic toward legitimate targets, and behave as regulatory cells capable of inhibiting the cytotoxic functions of other NK cells. This immediate functional inversion from an effector cell to a regulatory cell is directly due to acquired cell-surface HLA-G1[16]. We hypothesize that this mechanism could be related also to exosome sHLA-G release in the extracellular medium (Figure 2) for its re-capture, under the influence of unknown factors, may be chemokines and/or cytokines, but experimental investigations

are needed. Moreover, we hypothesize a direct role of HLA-G in affecting the IS value considering its direct inhibitory properties over NK cells and CTLs[130] (Figure 2). This topic could represent a matter of debate in the early future, not only for CRC.

HLA-G MODULATION REFLECTS 5'UTR AND 3'UTR GENETIC VARIABILITY

A growing body of evidence has been focusing in the last years on the 3'UTR polymorphisms and haplotypes, due to the microRNAs (miRNAs) interaction and their influence on expression. In particular, at least eight distinct haplotypes and eight SNPs have been described so far: the14-bp Insertion/Deletion (INDEL), +3003 T/C, +3010 C/G, +3027 C/A, +3035 C/T, +3142 C/G, +3187 A/G, and +3196 C/G[19] (Figure 1). INDEL and +3187 A/G SNPs have been associated with HLA-G mRNA stability and degradation processes even if, the exact mechanisms are not already been well elucidated[21,131]. In presence of the 14-bp Insertion (5'-ATTTGTTCATGCCT-3') sequence, HLA-G alleles have been associated with lower mRNA production[132]. In the other hand, it has been demonstrated that a smaller fraction of HLA-G mRNA transcripts presenting the 14-bp Insertion (Ins) can be alternative spliced from the mature HLA-G with the removal of 92 bases of exon 8 (include SNPs +3003 and +3010)[19]. mRNA producing smaller mRNA transcripts, reported to be more stable than the complete forms[20]. Interestingly, some AU-rich elements (ARE) are present in the 3'UTR of HLA-G, and it is known that these sequences are recognized by proteins causing rapid changes in mRNA stability[19,32]. The 14-bp sequence begins with AUUUG, and the absence of such motif in the 92 base-deleted transcripts might give an explanation of their resistance to degradation processes[20]. The presence of a guanine in the position +3142 increases the affinity of specific miRNAs (miR-148a, miR-148b and miR-152) to the HLA-G mRNA, decreasing HLA-G expression[32,133] (Figure 1). The influence of +3142G allele was demonstrated by functional studies in which HLA-G high expressing JEG-3 cells were transfected with miR-148a: decreased soluble HLA-G levels were detected[134].

In Table 1 are listed the associations found among HLA-G UTR SNPs and alleles, in particular the 14 bp INDEL polymorphism, and various disorders including cancer. Associations with circulating HLA-G levels were also reported if investigated. Recently, the risk of invasive cancer of uterine cervix was found to be significantly increased in presence of the 14 bp I/I and also the HLA-G*01:01:01:02 genotype in a large Canadian study of 539 women with histologically confirmed HG-CIN and invasive cancer; 833 women with normal cytology served as controls[33]. Moreover, 14 bp I/I genotype correlates with disease progression from high-grade cervical intraepithelial neoplasia (CIN3) to invasive cancer[33]. Previous data provided evidences of *HLA-G* expression in association with tumor metastasis and poor survival in an ovarian cancer animal model[135], and also with NK cell cytotoxicity inhibition and MMP-15 expression in ovarian cancer cell line[136]. In Kazakh population the risk of developing esophageal carcinoma was significantly higher in individuals carrying the 14 bp Del/Del (D/D) genotype[34], while Teixeira *et al*[35] showed that the 14 bp (D/D) genotype increases hepatocellular carcinoma (HCC) susceptibility in Brazilian population. Conversely, the 14 bp INDEL was no associated with HCC and liver cirrhosis susceptibility in a Korean study[137]. The 14 bp I/I was also found to be related to lower OS and DFS in patients with haematological malignancies undergoing allogeneic Hematopoietic Stem Cell Transplantation (allo-HSCT) and Methotrexate (MTX) treatment in univariate and multivariate analysis[138]. The 14 bp I/I genotype was also suggested to represent a therapy marker in Rheumatoid Arthritis (RA), able to identify responder patients treated with MTX[114].

To date, an association among the +3142 C>G SNP and a specific disease status, was recently described only in the Systemic Lupus Erythematous (SLE) autoimmune disorder[37] and in Relapsing-Remitting Multiple Sclerosis (RR-MS)[139] (Table 1).

A first attempt to find possible correlations between HLA-G genotype and phenotype (sHLA-G) was performed by Rebmann *et al*[140] in 2001, analyzing 94 healthy subjects. In particular, individuals carrying the HLA-G*01041 allele had significantly higher sHLA-G levels, while individuals with HLA-G*01031 allele and HLA-G*0105N allele, presented significantly lower of plasmatic and circulating HLA-G[140]. It should be noted that the 5'UTR and 3'UTR HLA-G regions were not investigated. The 14 bp I/I was then associated with significantly lower levels of sHLA-G in blood plasma or serum in different studies[141,142] (Table 1). Chen *et al*[143] reported a dramatic and significantly lower expression of sHLA-G in plasmatic samples from healthy donors (n = 150) of Chinese etnicity in the presence of the 14 bp I/I genotype with respect to the 14 bp D/D. Another recent investigation performed in MS patients in serum and cerebro spinal fluid (CSF), demonstrated an association among higher sHLA-G levels and +3142 C/C, 14 bp D/D genotypes and lower sHLA-G levels in +3142 G/G, 14 bp I/I combination[139] (Table 1).

Rizzo *et al*[144] identified a subgroup of Early Rheumatoid Arthritis (ERA) patients characterized by prevalence in 14 bp D/D, 14 bp D/I polymorphisms, and improved disease remission, therefore highlighting a protective role for the 14 bp Del allele. Moreover, the 14 bp Del allele was associated with higher sHLA-G and mHLA-G production and ITL2 expression. In 2010 Castelli *et al*[19] defined almost eight distinct 3'UTR haplotypes named from UTR-1 to UTR-8, that include the eight common polymorphisms of this nucleotide segment described above. Furthermore, HLA-G alleles were associated with each haplotype, and high Linkage Disequilibrium (LD) among most of the variants was observed, according to Hardy-Weimberg test. In particular, it was evidenced that the 14

bp Ins allele is always associated with the +3142G and +3187A alleles, both previously related with low mRNA availability, thus suggesting the implication of these two polymorphisms in lower mRNA production is associated with 14 bp insertion (Figure 1). The+3187G allele is only associated with the 3'UTR-1 haplotype carrying the 14 bp deletion[19].

It should be emphasized that 4-bp upstream the SNP +3187A/G there is an AUUUA motif, and 9-bp downstream to +3196C/G there is the presence of an UUAUUU motif. These (AU)-rich elements may modulate mRNA degradation and therefore expression level, and are also influenced by close sequence variations. Some authors, according to this nomenclature, started to analyze data from 3'UTR region in terms of haplotypes using different algorithm approaches such as EM algorithm, PHASE method, and FBAT. This haplotype analysis could represent an amazing way to correlate genetic data to the specific phenotypes of the study grouped also in a dominant or in a recessive model, or used to compare a single common haplotype with other phenotypes grouped together. To date, 3'UTR haplotypes analyses were performed in few studies and not regarded to cancer disease and so to CRC[113,131,145].

Recently, the functional impact of 3'UTR and in particular the 14 bp INDEL polymorphism, was deeply investigated by Svendsen *et al*[146], with particular attention on the processing and stability of the full-length membrane bound transcript of HLA-G1 (mHLA-G1). Authors, transducing different HLA-G1 DNA sequences in K562 human cell line, demonstrated that mRNA from 14 bp I/I had a higher degree of stability than the others, in accordance to the data reported in literature. Moreover, transductants carrying the 14 bp Ins, presented lower sHLA-G1 levels per mHLA-G1 ratio with respect to the constructs lacking the 14 bp Ins, but were the most efficient in inhibiting NK cytotoxicity[146].

In regard to the 5'UTR region, it has been less investigated and only recently, 16 SNPs in the 5'UTR region (Figure 1) and the 14-bp INDEL polymorphism in 3'UTR were analyzed in the same study in Brazilian patients who underwent assisted reproduction treatments (ART), characterized by failure implantation of embryos[113]. Larsen and Hviid[133], presented a complex panel of haplotypes related to the 5'UTR and in part of the 3'UTR regions showing clear LD between several of the polymorphisms centered around two main lineages of HLA-G alleles named G*010101$_{xx}$ and G*010102$_{xx}$, in accord to WHO classification. The HLA-G promoter region is considered unique among the *HLA* genes with many regulatory sequences, such as tissue specific regulatory element (TSRE) from position -1350 to -1100, and IFN-stimulated response element (ISRE) from -726 to -725 position[21,147]. The 5'UTR tri-allelic polymorphism -725C/G/T was evaluated in relation to plasma sHLAG concentration in a recent study in Iraqi women with recurrent pregnancy loss[148] A significantly association among lower levels of sHLAG in presence of the CC genotype was found *vs* the CG and CT condition[148] (Table 1). Of note, the presence in this position of a G nucleotide may alter the methylation profile of CpG di-nucleotides resulting in a modification of gene expression[113], and also influences binding of ISRE or other regulatory elements.

HLA-G IN CRC AND FUTURE APPLICATIONS

Despite the advances in knowledge and increasing interest in the immune contexture involvement, HLA-G has been poorly investigated in CRC and inflammation associated diseases of the gastrointestinal tract. A common mechanism present in CRC and in various types of cancer used by tumor cells to avoid recognition by CTLs, is the HLA class I down-regulation or total loss[149]. Altered HLA I class phenotypes regard reversible down regulation or irreversible mutational genes inactivation and are usually related to LOH of classical HLA-A, -B and -C heavy chains located in the chromosome region 6p21. HLA-G (protein and/or mRNA) is frequently over-expressed in tumors[16,17], including CRC[16,17,24-26] (Table 2). HLA-G expression was associated with malignant transformation and was never detected in the surrounding and closest areas near the tumor[17]. MHC class I loss or down-regulation due probably in defects or alterations in Processing Machinery (APM) components have been found in different malignancies and associated to reduced MHC class I recognition *vs* tumor-associated antigen (TAA)-specific CTL and disease progression[150]. LOH in the 15q21 region was observed in progressing lesions after immunotheraphy such as in melanoma[151]. Intriguingly, while classical HLA I proteins are frequently down regulate in in about 15% up to 75% of colon carcinoma lesions[150,152,153], HLA-G (related to isoform G1) results over-expressed in CRC malignant and pre-malignant tissues[26] (Table 2), in accord to his role in the host immune escape. Strong positive HLA-G expression was also detected in UD biopsies but no in tissues taken from patients affected by CD, thus it was proposed as a tool to better distinguish these inflammatory diseases[154] (Table 2).

LOH frequency for classical *HLA* genes in CRC was reported to be 40% evaluating 95 patients[155]. In CRC, higher LOH percentages were found in other chromosomal regions containing tumor suppressor genes *i.e.*, 43%-79% at 18q, 43%-76% at 17p and 17%-43% at 5q[156]. The irreversible total loss of HLA class I is generally referred to mutations affecting the β2m gene that are usually followed to loss of the second copy by LOH within his locus in the 15q21 region[157,158]. Expression of the β2m protein should be taken in consideration due its fundamental role in associate to HLAs molecules for correct antigen presentation. If β2m is lost, stable antigen-HLA class I complexes cannot be produced[156]. Of note, the major function of HLA-G is not antigen presentation and, if β2m is necessary to assemble the most studied HLA-G1, sHLA-G1 and sHLA-G5 isoforms, it should

be discussed the function of alternative spliced isoforms lacking β2m. We speculate that HLA-G alternative spliced isoforms without β2m assembly could represent another way to escape from immune control, especially in tumor such as CRC in which β2m downregulation or loss is a frequent event[158]. Moreover, their role should be established and explored (which are their interactors?) providing new insides of the HLA-G planet.

Alterations due to LOH have been reported in 25%[159]-35%[156] of CRCs in β2m 15q21 and in 6p21 in 40% of the same patients[156], demonstrating a strong correlation that may impact on disease progression in terms of immune escape exert by the tumor[160]. To date in CRC, HLA-G polymorphisms and haplotypes have not been investigated to find correlations with prognosis or phenotype (circulating sHLA-G proteins) even if, recently, some authors started to report complex and intriguing analysis not related to cancer[147,148].

Data about the soluble HLA-G has been reported only for CRC patients of Chinese ethnicity[27,28] suggesting that sHLA-G should be considered as a good diagnostic tool superior to classical CEA[27], and also a useful indicator to distinguish benign colorectal related disease from CRC. Of note, Cao *et al*[28] collected and analyzed plasma samples from a limited patient series, while Zhu *et al*[27] quantified sHLA-G in a quite large cohort of patients in serum that is not the recommended biological sample (Table 2). Both authors did not genotype patients at the germinal level to search for a possible phenotype correlation and/or to assess specific HLA-G alleles and genotypes related to CRC. No correlations were performed with clinical outcome of CRC patients in terms of survival, disease relapse and response to therapy treatment. In our opinion, detection of sHLA-G is not sufficient alone and should be associated to genotyping of the gene, with particular attention on the regulatory untranslated regions that are susceptible to post translational modifications such as methylation, transcription factors and miRNA binding (Figure 1). Moreover, sHLA-G assay should be standardized and defined by precise guidelines to improve its clinical application. Recently, preliminary data that need further investigations, showed that higher sHLA-G expression in mucor secretory *vs* non-mucosecretory CRC, correlate with worse prognosis of patients[128].

A differential spontaneous sHLA-G secretion from PBMC was reported in CD (high levels of the secrete molecules) and a lack of sHLA-G in UC also after inflammatory stimulus by Lipopolysaccharide (LPS) activation[161] (Table 2). Conversely, opposite results were previously described analyzing HLA-G protein expression by IHC in CD and UC biopsies, with higher expression levels in UC and negative staining in CD samples[154] (Table 2). Subsequently, these controversial data were not confirmed with novel studies. However, authors who reported a lack of sHLA-G in UC[162], showed that immunosuppressive drugs influence the sHLA-G secretion in UC and CD patients, stimulating its release in UC and decrease it in CD[162] (Table 2). Halama *et al*[163], basing on the evidence that CRC patient prognosis is dependent on the local immune contexture, characterized NK and T cells localization and densities in primary CRC liver metastasis, adenomas and normal tissues. NK cells were rare in tumor tissue independently of HLA class I expression, and also not depending from chemokine levels that were rather elevated and correlated to T cells infiltration[163] Subsequently, for the first time Rocca *et al*[164], characterized tumor-associated NK cells (TANKs), with respect to autologous peripheral blood NK cells (PB-NKs), from CRC patients, and compared the latter with PB cells from healthy donors. Authors demonstrated an altered phenotype for TANKs in CRC patients, with a low expression of activating receptors and also with an impaired degranulation and release of cytokines (IFN-γ) capacity. It should be pointed that HLA-G evaluation would be of interest in both these analysis, especially considering recent data about sHLA-G role in impair NK cells by (1) modulation of specific chemokine secretion (CXCR3, CX3CR1, and CCR2); (2) functional inhibition on CD94/NKG2A receptor; and (3) modulation of NK chemokines and cytokines secretion[165].

Finally, the host-immune reaction could be the critical element in determining response to therapy, and the effect on the immune response could be the underlying factor behind many of the predictive markers[13]. In ovarian cancer, positive HLA-G cells from peritoneal and pleural effusions decrease in number after chemotherapy and this result correlates with improved survival of the related patients[166]. These data suggest that HLA-G-expressing cells are more susceptible to elimination by the immune response or treatment[162,166].

On the opposite, IFN-α immunotherapy showed to further increase circulating sHLA-G levels in melanoma patients[30], thus in accord to the presence of ISRE element in the 5'UTR region[21]. It is of knowledge that common therapies in cancer (chemotherapy and radiotherapy) have an impact on immune system[54], that could be different depending from the therapy regiment, often associated to different grades of toxicity and neutropenia[167]. A favourable prognosis correlated with TILs in stage Ⅲ CRC patients treated by surgery alone (n = 851) or 5-fluorouracil (5-FU) adjuvant chemotherapy (n = 305), was observed[168]. Moreover, high densities TILs in metastatic liver lesions at the invasive margin revealed strong association with chemotherapy efficacy and prognosis in advanced CRC patients[169,170]. These studies based on infiltrating lymphocytes in correlation with therapy, have provided the scientific basis to implement the concept of IS and, in the future, we hope that IS validation will provide a well defined tool to assess CRC prognosis and clinical outcome based also on patient treatment. Due to HLA-G functional properties in the direct inhibition of cytotoxic and memory T lymphocytes, we stress the possible association between IS and HLA-G (Figure 2) and therefore patient prognosis.

Moreover, particular HLA-G haplotypes and/or genotypes and allelic variants could be identified as predictive

genetic marker of response treatment, to better stratify patients.

In particular, further investigations in large cohort of patients are needed to define: HLA-G haplotypes, genotypes, and alleles frequencies and association with: (1) the risk of CRC (possible distinctions comparing them with those of UD and CD diseases); (2) the prognostic power: relation with OS, DFS, and predictive value of response to therapy; (3) plasma sHLA-G levels as predictive and prognostic markers; and (4) genotype-phenotype correlations among soluble and HLA-G genotypes/alleles (especially 5'UTR and 3'UTR nucleotide variations).

We suggest that HLA-G could represent a novel prognostic immune biomarker with a prominent role in inhibiting host immune response in CRC. We also suggest to assess an association with IS and HLA-G (Figure 2) to better characterize host immune response and complete the immune contexture overview in CRC patients.

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