Clinical relevance of miR-mediated HLA-G regulation and the associated immune cell infiltration in renal cell carcinoma

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Abbreviations: ACTB, β-actin; APM, antigen processing machinery; B2-m, β-2-microglobulin; β-gal, β-galactosidase; B7-H1, B7 homolog 1; CDS, coding sequence; Cr, chromium; COPZ2, coatomer protein complex, subunit zeta 2; DAC, 5′-aza-2′-desoxycytidine, GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HLA-G, human leukocyte antigen G; HRP, horseradish peroxidase; IHC, immunohistochemistry; IL, interleukin; ILT, immunoglobulin-like transcript; IFNγ, interferon gamma; LAK, lymphokine-activated killer cell; luc, luciferase; mAb, monoclonal antibody; MDSC, myeloid-derived suppressor cells; MFI, mean-specific fluorescence intensity; miR, microRNA; miTRAP, miRNA trapping by RNA in vitro affinity purification; n.d., not determined; NK, natural killer cell; n.o.s., not otherwise specified; ntc., non-template control; sHLA-G, soluble HLA-G; SNP, single nucleotide polymorphism; RCC, renal cell carcinoma; TGF-β, transforming growth factor β; TIL, tumor infiltrating lymphocyte; TMA, tissue microarray; Treg, regulatory T cell; UTR, untranslated region; WB, Western blot analysis; WT, wild type

In human tumors of distinct origin including renal cell carcinoma (RCC), the non-classical human leukocyte antigen G (HLA-G) is frequently expressed, thereby inhibiting the cytotoxic activity of T and natural killer (NK) cells. Recent studies demonstrated a strong post-transcriptional gene regulation of the HLA-G by miR-152, −148A, −148B and −133A. Standard methods were applied to characterize the expression and function of HLA-G, HLA-G-regulatory microRNAs (miRs) and the immune cell infiltration in 453 RCC lesions using a tissue microarray and five RCC cell lines linking these results to clinical parameters. Direct interactions with HLA-G regulatory miRs and the HLA-G 3′-untranslated region (UTR) were detected and the affinities of these different miRs to the HLA-G 3′-UTR compared. qPCR analyses and immunohistochemical staining revealed an inverse expression of miR-148A and −133A with the HLA-G protein in situ and in vitro. Stable miR overexpression caused a downregulation of HLA-G protein enhancing the NK and LAK cell-mediated cytotoxicity in vitro. CD107a activation assays revealed a HLA-G-dependent cytotoxic activity of immune effector cells. A significant higher frequency of CD3+/CD8+ T cell lymphocytes, but no differences in the activation markers CD69, CD25 or in the presence of CD56+ FoxP3+ and CD4+ immune cells were detected in HLA-G+ compared to HLA-G− RCC lesions. This could be associated with higher WHO grade, but not with a disease-specific survival. These data suggest a miR-mediated control of HLA-G expression in RCC, which is associated with a distinct pattern of immune cell infiltration.

Introduction

Although the non-classical HLA-G exerts some similarities to the classical HLA class Ia molecules, it differs from those in regard to its limited allelic variability, the existence of seven isoforms generated by alternative splicing and its restricted physiologic expression to mainly immune-privileged tissues, e.g. cytotrophoblasts, but also organs sustaining erythropoiesis.1,2 HLA-G can counteract immune cell activation by its interaction with inhibitory lymphocyte receptors like the immunoglobulin-like transcript (ILT)2, ILT4 and the killer immunoglobulin-like receptor KIR2DL4 on NK, T, B, dendritic cells (DC) and neutrophils.3,4 In addition, HLA-G induces apoptosis of activated CD8+ T cells and suppresses CD4+ T cell proliferation in response to allogenic stimulation.7,9 HLA-G expression is controlled at the epigenetic, transcriptional, post-transcriptional as well as post-translational level.10 DNA methylation and hypoacetylation of the histones H3 and...
H4 represent a regulatory process of HLA-G expression.\textsuperscript{11} Despite its low polymorphism when compared to classical HLA class I antigens, there exists a high frequency of nucleotide variability in the promoter as well as in the 3' untranslated region (3'-UTR) of HLA-G. Furthermore, the 3'-UTR of HLA-G has been suggested as a target for several miRs. miRs represent a class of non-coding RNA molecules with approximately 22 nucleotides in length and regulate sequence-specific gene expression by binding preferentially to the 3'-UTR of mRNAs leading to translational inhibition and/or degradation of the target mRNA.\textsuperscript{12-14}

Under pathophysiologic conditions, constitutive HLA-G expression could be frequently found in both haematopoietic and solid tumors including RCC,\textsuperscript{15} which could be correlated in some tumor entities with an unfavorable prognosis and a poor clinical outcome of patients. In addition, high levels of soluble HLA-G have been detected in sera and ascites of tumor patients, which correlated with an advanced disease status and high tumor load and thus might represent a marker for tumor prognosis.\textsuperscript{16-18} The discordant HLA-G mRNA and protein expression in tumors suggest a post-transcriptional gene regulation of HLA-G. Indeed, members of the miR-148 family and miR-133A have been recently identified to target the 3'-UTR of HLA-G.\textsuperscript{13,14,19-21} Their biologic activity seemed to be sufficient to control HLA-G expression levels under physiologic conditions thereby increasing NK cell-mediated killing \textit{in vitro}.

Despite miR-148 family members might exert tumor suppressive activity,\textsuperscript{22-31} there exists no information about the clinical relevance of these miRs and their correlation to HLA-G expression in RCC. To characterize the underlying mechanisms of the post-transcriptional control of HLA-G in tumors, a set of human RCC cell lines and lesions was analyzed for their HLA-G transcript, protein and miR expression pattern. A direct interaction between HLA-G-specific miRs and the HLA-G 3'-UTR was confirmed by an miR-enrichment assay termed miTRAP.\textsuperscript{34} The functional consequences of HLA-G-specific miRs were established by generation of stable miR transfectants followed by determination of HLA-G expression levels and immune recognition. Furthermore, the clinical relevance of HLA-G and HLA-G-specific miRs and their association with the immune cell infiltration of RCC lesions was investigated by immunohistochemical analysis of tissue microarrays (TMA) and correlated to the clinicopathologic parameters of the patients.

**Results**

**Expression pattern of candidate miRs controlling HLA-G in RCC cell lines**

Although the miR-148 family members miR-148A/B and miR-152 as well as the miR-133A have been suggested to target the 3'-UTR of HLA-G and affect NK cell-mediated cytotoxicity,\textsuperscript{14,21,32,33} the role of these miRs in tumors including RCC has not yet been investigated. Therefore, the expression profiles of the four miRs were first analyzed in RCC cell lines and correlated to the HLA-G expression levels. As representatively shown in Figure 1A for some selected model systems, RCC cell lines expressed heterogeneous HLA-G transcript levels as well as different splicing patterns when compared to JEG-3 cells, which serve as positive control. Despite HLA-G transcript was detectable in two (MZ2733RC and MZ2905RC) of five RCC cell lines, the corresponding protein was only detectable in RCC cell line MZ2733RC (Fig. 1B).

In addition, a heterogeneous expression pattern of a HLA-G-specific miR panel was detected in the RCC cell lines analyzed (Fig. 1C): The HLA-G mRNA\textsuperscript{+}/protein\textsuperscript{+} MZ2905RC cells showed the highest miR-152 expression, while the HLA-G mRNA\textsuperscript{+}/protein\textsuperscript{+} JEG-3 cells displayed an approximately 1,000-fold lower miR-152 expression level when compared to the RCC cell lines. Interestingly, the HLA-G mRNA\textsuperscript{+}/protein\textsuperscript{+} RCC cell line MZ2733RC exhibited a 100-fold reduced expression of miR-148A compared to all other analyzed cell lines, whereas miR-148B was only marginally expressed in the cell systems. However, the miR-133A showed no difference in their expression in the analyzed cell lines. The HLA-G non-relevant miR-141 and miR-541 serving as internal controls were also quantified. While miR141 was moderately expressed in all cell systems, miR-541 was not detectable. Noteworthy, HLA-G protein and miR152 and miR-148A expression levels in the JEG-3 and in the 2 RCC cell lines (MZ2733RC and MZ2905RC) were inversely correlated. The direct interaction between the reported HLA-G regulatory miRs with the 3'-UTR of HLA-G was investigated by their specific enrichment from a cell lysate of the HLA-G mRNA\textsuperscript{+}/protein\textsuperscript{+} RCC cell line MZ2905RC using the miTRAP technique.\textsuperscript{34} Employing the \textit{in vitro} transcribed HLA-G 3'-UTR as a bait, an enrichment of all members of the miR-148 family as well as of miR-133A was found, whereas the HLA-G non-relevant miR-141 was present in the Input could not be enriched (Fig. 1D). The affinity of the miRs relevant for the HLA-G 3'-UTR can be defined as follows: miR-152 > miR-148A > miR-148B > miR-133A. Therefore, a quotient (enrichment factor) of specifically enriched miRs applying the HLA-G 3'-UTR as bait and unspecific enriched miRs using a mock sequence of four MS2 loops as bait was calculated.

To compare the affinity of HLA-G regulatory miRs to the HLA-G 3'-UTR, the miTRAP experiment has a preference to experiments of overexpressing miRs by plasmids or transiently transfection of precursor-miR constructs, which is based on the fact that a correct processing of the miR constructs is required for their biological function and this processing is affected by the sequence of the miR flanking regions. These miR specific effects could negatively influence the results of such reporter gene experiments as comparison of the affinity of different miRs to one target.

The miR-152 showed by far the highest affinity for the HLA-G 3'-UTR and was therefore overexpressed in HLA-G\textsuperscript{+} cells to investigate the immune modulatory potential of HLA-G \textit{in vitro}.

**Effects of HLA-G-specific miRs on the immune response**

As reported in Manaster et al.,\textsuperscript{20,34} the JEG-3 cells are an inappropriate model system to investigate a HLA-G-dependent cytotoxicity of immune effector cells. In order to determine the influence of the miR-152-mediated silencing of HLA-G on the
immune effector cell-mediated cytotoxicity, a murine model system based on NIH/3T3 cells transfected with HLA-G (CDS and 3’-UTR) and/or respective miR expression vector was generated (Fig. 2A). The use of these cell systems was possible since the endogenously expressed miR-152 of murine and human origin exert sequence homology (www.mirbase.org). NIH/3T3 cells lack the expression of putative other inhibitory molecules for human immune effector cells. Stable HLA-G transfectants of NIH/3T3 cells displayed higher HLA-G protein expression levels when compared to HLA-G-negative JEG-3 cells (Fig. 2A). The co-expression of miR-152 in HLA-G-positive NIH/3T3 cells resulted in a downregulation of HLAG-specific mRNA, which was accompanied by a total loss of HLA-G protein in these cells. The expression of human HLA-G protects the murine cells from the lysis by human NK and LAK cells (Fig. 2B and C). Nevertheless, the
miR-152-mediated downregulation of HLA-G in the transfectants at least partially restored their sensitivity to LAK and NK cell-mediated lysis, demonstrating the immune modulatory effect of HLA-G in vitro.

Inverse expression of HLA-G and HLA-G regulatory miRs in RCC lesions and their clinical relevance

The frequency of HLA-G expression was assessed in RCC lesions using a TMA consisting of 453 RCC lesions and matched normal kidney epithelium. 216 of 433 (49.9%) RCC samples showed a membranous, 165 of 433 (38.1%) a cytoplasmic expression of HLA-G, but the staining intensity strongly varied (Table 3). Indeed, cytoplasmic HLA-G staining is associated to WHO grade ($p = 0.014$). Grade 3 tumors more frequently demonstrate strong cytoplasmic HLA-G staining than those of lower grade (Table 3). To further assess whether the inverse correlation between the expression levels of HLA-G and its regulatory miRs detected in vitro also exists in situ 36 selected HLA-G$^+$ and 36 HLA-G$^-$ RCC lesions were monitored for miR-133A, miR-148A, miR-148B and miR-152 expression. The expression levels of miR-133A and miR-148A, but not of miR-152 were significantly (for miR-133A almost significantly) decreased in HLA-G$^+$ RCC lesions when compared to HLA-G$^-$ RCC lesions (Figs. 3 A–E). Referring to the data obtained from the RCC cell lines, the miR-148B was only barely detectable in HLA-G$^-$ and HLA-G$^+$ RCC lesions (data not shown).

Many recent studies demonstrated the tumor suppressive function of the miR133A and the miRs of the miR-148 family.22,24,28,29,31,46,54,57,58 This inverse correlation of lowered miR-133A and miR-148A expression levels and higher HLA-G staining intensity was for both miRs. High HLA-G protein levels and the reduced miR-133A/miR-148A expression can be linked to each other and to the WHO grade.

Correlation of the immune cell infiltration with HLA-G expression in RCC

The infiltration of immune effector cells in RCC lesions was determined in the 72 selected RCC lesions (Table 1) using antibodies directed against CD3, CD4, CD8, CD56, FoxP3 and the activation markers CD69 and CD25. As shown in Figure 4 and respectively in Supplemental Figure 1, the 36 HLA-G$^+$ RCC lesions had a statistically significant higher frequency of CD3$^+$...
Table 1. Characteristics of the 72 RCC lesions (36 HLA-G\(^{-}\) and 36 HLA-G\(^{+}\)) analyzed for miR-148A, miR-152 and miR-133A

| Tumor sample | HLA-G protein | Membranous "staining intensity (percentage of positive tumor cells)" | Cytoplasmic "staining intensity (percentage of positive tumor cells)" | RCC Subtype | WHO grade | Metastases |
|--------------|---------------|-------------------------------------------------|-------------------------------------------------|-------------|-----------|-----------|
| I 1          | 0(0)          | 0(0)                                             | Chromophobe                                     | G2          | n.d.      |           |
| I 3          | 0(0)          | 0(0)                                             | Chromophobe                                     | G2          | n.d.      |           |
| I 5          | 0(0)          | 0(0)                                             | Clear cell                                      | G2          | n.d.      |           |
| I 6          | 0(0)          | 0(0)                                             | Clear cell                                      | G1          | n.d.      |           |
| I 7          | 0(0)          | 0(0)                                             | Clear cell                                      | G3          | n.d.      |           |
| I 9          | 0(0)          | 0(0)                                             | Chromophobe                                     | G2          | n.d.      |           |
| I 10         | 0(0)          | 0(0)                                             | Clear cell                                      | G2          | n.d.      |           |
| I 12         | 0(0)          | 0(0)                                             | Chromophobe                                     | G2          | n.d.      |           |
| I 14         | 0(0)          | 0(0)                                             | Clear cell                                      | G2          | n.d.      |           |
| I 26         | 0(0)          | 0(0)                                             | Chromophobe                                     | G2          | n.d.      |           |
| I 32         | 0(0)          | 0(0)                                             | Clear cell                                      | G2          | n.d.      |           |
| I 41         | 0(0)          | 0(0)                                             | Papillary/clear cell                            | n.d.        | n.d.      |           |
| I 42         | 0(0)          | 0(0)                                             | Chromophobe                                     | G2          | n.d.      |           |
| I 51         | 0(0)          | 0(0)                                             | Clear cell                                      | G2          | n.d.      |           |
| I 56         | 0(0)          | 0(0)                                             | Clear cell                                      | G2          | n.d.      |           |
| I 59         | 0(0)          | 0(0)                                             | Papillary/clear cell                            | n.d.        | n.d.      |           |
| II 2         | 0(0)          | 0(0)                                             | Clear cell                                      | G2          | n.d.      |           |
| II 5         | 0(0)          | 0(0)                                             | Clear cell                                      | G2          | no        |           |
| V 13         | 0(0)          | 0(0)                                             | Clear cell                                      | G3          | n.d.      |           |
| V 15         | 0(0)          | 0(0)                                             | Clear cell                                      | G2          | n.d.      |           |
| V 19         | 0(0)          | 0(0)                                             | Chromophobe                                     | G2          | n.d.      |           |
| V 20         | 0(0)          | 0(0)                                             | Clear cell                                      | G3          | yes       |           |
| V 21         | 0(0)          | 0(0)                                             | Clear cell                                      | G2          | n.d.      |           |
| V 28         | 0(0)          | 0(0)                                             | Clear cell                                      | G2          | n.d.      |           |
| V 35         | 0(0)          | 0(0)                                             | Clear cell                                      | G3          | n.d.      |           |
| V 54         | 0(0)          | 0(0)                                             | Clear cell                                      | G2          | no        |           |
| VI 3         | 0(0)          | 0(0)                                             | Clear cell                                      | G2          | no        |           |
| VI 5         | 0(0)          | 0(0)                                             | Clear cell                                      | G3          | no        |           |
| VI 7         | 0(0)          | 0(0)                                             | Sarcomatoid                                     | G3          | no        |           |
| VI 8         | 0(0)          | 0(0)                                             | Clear cell                                      | G2          | no        |           |
| VI 9         | 0(0)          | 0(0)                                             | Clear cell                                      | G2          | no        |           |
| VI 10        | 0(0)          | 0(0)                                             | Chromophobe                                     | G2          | no        |           |
| VI 12        | 0(0)          | 0(0)                                             | Clear cell                                      | G2          | no        |           |
| VI 13        | 0(0)          | 0(0)                                             | Clear cell                                      | G2          | no        |           |
| VI 20        | 0(0)          | 0(0)                                             | Clear cell                                      | G3          | no        |           |
| I 8          | 2(100)        | 2(80)                                            | Clear cell                                      | G3          | n.d.      |           |
| I 25         | 3(100)        | 2(80)                                            | Clear cell                                      | G2          | n.d.      |           |
| I 29         | 3(100)        | 2(100)                                           | Clear cell                                      | G2          | n.d.      |           |
| I 37         | 3(100)        | 2(90)                                            | Clear cell                                      | G2          | n.d.      |           |
| I 44         | 3(100)        | 3(100)                                           | Clear cell                                      | G3          | n.d.      |           |
| I 46         | 3(100)        | —                                                | Papillary/clear cell                            | G2          | n.d.      |           |
| II 4         | 3(100)        | 2(80)                                            | n.o.s                                           | n.d.        | n.d.      |           |
| II 10        | 3(100)        | 1(100)                                           | Clear cell                                      | G3          | no        |           |
| II 39        | 3(100)        | 2(100)                                           | Clear cell                                      | G2          | no        |           |
| II 58        | 3(80)         | 2(40)                                            | Clear cell                                      | G2          | n.d.      |           |
| III 2        | 3(100)        | 2(100)                                           | Clear cell                                      | G3          | n.d.      |           |
| III 11       | 3(80)         | 2(80)                                            | Clear cell                                      | G3          | n.d.      |           |
| III 20       | 3(100)        | 2(50)                                            | Clear cell                                      | G3          | n.d.      |           |
| III 31       | 3(100)        | 2(40)                                            | Clear cell                                      | G2          | yes       |           |
| III 33       | 3(90)         | 3(90)                                            | Clear cell                                      | G2          | n.d.      |           |
| III 36       | 3(90)         | 2(90)                                            | Clear cell                                      | G3          | n.d.      |           |
| III 39       | 3(90)         | 3(50)                                            | Clear cell                                      | G3          | n.d.      |           |
| III 45       | 3(100)        | 2(80)                                            | Clear cell                                      | G2          | n.d.      |           |
| IV 2         | 3(100)        | 3(100)                                           | Clear cell                                      | G3          | yes       |           |
| IV 10        | 3(100)        | 2(80)                                            | Clear cell                                      | G2          | n.d.      |           |
| V 16         | 3(100)        | —                                                | Clear cell                                      | G1          | n.d.      |           |

(Continued on next page)
and CD8⁺ T cells \( (p < 0.001) \), but not of CD4⁺ or CD56⁺ cells. While the NK, NKT and CD4⁺ T cell infiltration did not vary, a significant difference in CD3⁺ \( (p < 0.001) \) and CD8⁺ cytotoxic T cells (CTL; \( p < 0.001 \)) between HLA-G⁺ and HLA-G⁻ RCC lesions was detected. The frequency of CD4⁺ (Fig. 4B) and FoxP3⁺ (data not shown) T cells was low and independent of HLA-G expression.

The activity of the CD3⁺ and CD8⁺ CTL was determined by staining of CD69 and CD25 activation markers. A higher, but not statistically significant number of CD69⁺ cells \( (p D 0.08) \) was found in HLA-G⁺ tumors (Fig. 4B). In contrast, CD25⁺ T cells were barely detectable in all RCC lesions independent of HLA-G status. The increased frequency of CD3⁺ and CD8⁺ T cells in HLA-G⁺ tumors did correlate with HLA-G expression (staining intensity) and therefore also with a higher WHO grading and further strengthens the immune modulatory potential of HLA-G.

**Correlation between the presence of tumor-infiltrating lymphocytes and the disease-specific survival of RCC patients**

To address whether HLA-G expression (staining intensity) and/or the frequency of tumor-infiltrating lymphocytes (TILs) of RCC lesions contribute to survival of RCC patients, the immunohistochemistry (IHC) data of HLA-G, CD3, CD8, CD4, CD56, CD69, CD25 and FoxP3 were tested for correlations with disease-specific survival (Fig. 5).

HLA-G expression had almost no effect \( (p = 0.67) \) on the disease-specific survival of RCC patients. Neither HLA-G expression, CD69, CD56, CD3, CD4, CD8, CD25 nor FoxP3 expression demonstrated significant correlations to disease-specific survival of RCC patients. Only the presence of CD4⁺ cells appear to contribute to a better disease-specific survival, but which was not statistically significant \( (p = 0.097; \text{Fig. 5}) \).

**Discussion**

HLA-G expression has been demonstrated in RCC cells at a high frequency, thereby protecting tumor cells from immune cell-mediated cytotoxicity. However, RCC and other tumor...
cells often show a discordant expression of HLA-G mRNA and protein suggesting a post transcriptional gene regulation of HLA-G by e.g. HLA-G-specific miRs. This concept was further supported by the recent identification of miR-148A, miR-148B, miR-152 and miR-133A directly targeting the 3'-UTR of HLA-G.\textsuperscript{14,21,33,36} Since these miRs are reported to control HLA-G expression, the current study explored the nature of these interactions by analyzing the binding characteristics of these miRs to HLA-G mRNA, their tissue-specific expression pattern and functional consequences. In addition, HLA-G protein levels

### Table 3. Correlation of HLA-G expression with WHO grade

| HLA-G expression  | WHO grade G1 [%] | WHO grade G2 [%] | WHO grade G3 [%] |
|-------------------|------------------|------------------|------------------|
| HLA-G membranous  |                  |                  |                  |
| HLA-G negative    | 13.4             | 63.6             | 23.0             |
| HLA-G weak        | 16.2             | 71.6             | 12.2             |
| HLA-G moderate    | 17.1             | 63.2             | 19.7             |
| HLA-G strong      | 10.6             | 56.1             | 33.3             |
| HLA-G cytoplasmic |                  |                  |                  |
| HLA-G negative    | 17.5             | 62.3             | 20.1             |
| HLA-G weak        | 8.6              | 69.1             | 22.2             |
| HLA-G moderate    | 6.2              | 70.8             | 23.1             |
| HLA-G strong      | 15.8             | 36.8             | 47.4             |

Distribution of membranous and cytoplasmic HLA-G expression over different categories of WHO grade in RCC tumors, as found in HLA-G immunohistochemistry. Only staining intensity is shown. Strong cytoplasmic expression significantly accumulates in G3 tumors ($p = 0.014$).

Figure 3. Inverse correlation of HLA-G and HLA-G relevant miR expression in RCC tumor lesions. (A) Representative immunohistochemical staining for a HLA-G$^-$ RCC lesion (sample II5, WHO grade: G2). (B) Representative immunohistochemical staining for a HLA-G$^+$ RCC lesion (sample II39, WHO grade: G2). (C, D, E) Quantification of the expression of miR-148A (C), miR-152 (D) and miR-133A (E) by qPCR in 36 HLAG$^-$ and 36 HLAG$^+$ RCC lesions (listed in Table 1), respectively. The absolute copy number determination is visualized as Box–Whisker plot. Statistical analyses were performed as described in Materials and Methods.
were correlated with the HLA-G-specific miRs, immune cell infiltration and WHO grade.

The direct interaction between the miR-148 family members and the HLA-G 3'-UTR was confirmed in RCC cells using the miTRAP technology demonstrating a strong enrichment of miR-152 and to a lesser extent of miR-148A when compared to controls, which might be due to their lower constitutive expression levels in the RCC cell line used for cell lysate (MZ2905RC). Surprisingly, miR-133A was very weak enriched. Indeed, Wang et al., 2012\cite{21} report that miR-133A does not affect HLA-G mRNA but reduces HLA-G protein level if overexpressed in JEG-3 cells.

Since JEG-3 cells have been demonstrated as a not valuable model for analyzing the HLAG-dependent cytotoxicity of immune effector cells. Murine NIH/3T3 cells overexpressing HLA-G (CDS & 3'-UTR)
were generated. Since HLA-G could be detected as β2-m-free dimers or even trimers on the cell surface\cite{37,38} the lack of human β2-m for human MHC class I molecules is not interfering with cytotoxicity. Transfection of HLA-G expressing NIH/3T3 cells with miR-152 strongly down regulates HLA-G expression, which is in line with reports describing the expression and processing of human miRs in murine NIH/3T3 cells.\cite{39,40} While HLA-G expressing NIH/3T3 cells were completely resistant to NK or LAK cell-mediated cytotoxicity, miR-152 overexpression caused a partial restoration of LAK cell activity suggesting that miR-152 could alter the HLA-G-induced tolerogenic phenotype. In addition, immunohistochemical staining of a TMA of RCC lesions with an anti-HLA-G monoclonal antibody (mAb) demonstrated a high frequency of HLA-G\textsuperscript{+} tumor lesions, which was associated with an increased WHO grade, but not with disease-specific survival of RCC patients. This was accompanied by a significantly reduced expression of miR-148A and almost significantly reduced ($p = 0.052$) miR-133A, but not of miR-152 in HLA-G\textsuperscript{+} tumor lesions suggesting a prognostic potential

Figure 5. Correlation between the presence or absence of TILs and the HLA-G expression with disease-specific survival of the patients visualized as Kaplan–Meier-Plots. Kaplan–Meier-Plots of disease-specific survival in relation to HLA-G expression and tumor-infiltrating lymphocytes. The presence of CD4\textsuperscript{+} cells was associated (but not significant; $p = 0.097$) with better disease-specific survival. The markers HLA-G, CD3, CD8, CD56, CD4, FOXP3, CD 69 and CD 25 showed no significant correlation to disease-specific survival.
of these two miRs. Furthermore, differences in the immune cell infiltration between HLA-G+ and HLA-G− RCC lesions exist, which were only significant for CD3+ and CD8+ cells, but had no impact on patients outcome and might be due to the HLA-G-induced tolerogenic phenotype of HLA-G+ RCC. Indeed, Liu and coworkers (2014) report that increased T cell infiltration in primary RCC tumors can be associated with worse clinical outcome. Maybe our data can explain such observations, due to the correlation of HLA-G expression and higher grading of the RCC tumors on the one hand and on the other one the downregulated tumor suppressive miRs miR-148A and miR-133A and the higher infiltration of mostly inactive CD3+CD8+ T cells.

However, HLA-G+ RCC lesions exert a very limited infiltration by regulatory T cells (Tregs) as determined by the number of CD25+ and FoxP3+ cells. The presence of CD3−CD4+ cells could be correlated to a better disease-specific survival, but not significantly (p = 0.097). Thus, CD4+ cells in the RCC lesions might represent granulocytes, macrophages or DCs. Further studies should investigate the influence of CD3−CD4+ immune cells in RCC tumor lesions. In this context, it is noteworthy that HLA-G+ gastric tumors were shown to be associated with a significant poorer patients’ survival, which is accompanied by an increased number of Tregs.

The miR-148A gene is located on the short arm of chromosome 7 (7p15.2), the miR-133A on the long arm of chromosome 18 (18q11.2), and a second miR-133A gene is located at the long arm of chromosome 20 (20q13; www.mirbase.org), but so far there exists no common chromosomal defect of the region 7p15.2 in RCCs but for 18q a significant poorer patients’ survival, which is accompanied by an increased number of Tregs.

Instead, RCCs often show polysomy for chromosome 7(2,4,44) or duplication of the region 20q. Interestingly, it was recently published that the miRs of the miR-148 family could be silenced upon hypermethylation. Thus, the underlying mechanisms of miR-148A downregulation in RCC have still to be investigated in more detail. Since lack of HLA-G expression in tumors is often epigenetically controlled, one might consider an inverse methylation status of HLA-G and HLA-G-specific miRs.

However, the members of the miR-148 family and miR-133A might serve as potential diagnostic markers and provide a novel treatment strategy for tumors, which is in line with its reported tumor suppressor activity. Further studies are required to support the tumor suppressive function of the miR-148A family and miR-133A in vivo by inhibiting the tumor immune escape mediated by HLA-G.

**Methods**

**DNA isolation**

DNA was isolated from different human cell lines using the QIAamp DNA Mini Kit (Qiagen, Cat. no. 51304) according to the manufacturers’ protocol.

**RNA and miR isolation, semi-quantitative and quantitative PCR**

Total RNA from cell lines was isolated with the TRIzol Reagent (Invitrogen, Cat. no. 15596–026) according to the manufacturers’ instructions. The RNA was treated with DNeasy I (NEB, Cat. no. M0303S). For RNA isolation from paraffin-embedded tissue sections, total RNA was extracted using the MasterPure Complete DNA and RNA Purification Kit (Epicentre Biotechnologies, Cat. no. MC85200) according the manufacturers’ protocol.

RNA was reverse transcribed into cDNA using the ReverTaidTM H Minus First Strand cDNA synthesis kit (Fermentas, Cat. no. K1631). For miR-specific cDNA synthesis, a miR-specific stem-loop primer altered was used (1,47,48) while for reverse transcription of mRNA oligo dT primers (Fermentas) or the HLA-G-specific reverse primer (5′-TGAGACAGAGCGGAGACAT-3′) were employed. For semi-quantitative RT-PCR, the Taq DNA polymerase kit (Invitrogen, Cat. no. 10342–020) and for qPCR the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Cat. no. 11733–038) were applied.

The reactions were run as triplicates of biologic replicates. For qPCR of miRs, the absolute copy numbers were determined against an external miR-specific TOPO-TA plasmid standard (Invitrogen, Cat. no. K4510–22). The miR-specific TOPO-TA plasmids were generated by cloning the respective stem-loop PCR product into this plasmid. All oligonucleotides used for mRNA and miR expression profiling are listed in the Table 4.

**Tissue microarray and immunohistochemistry**

TMA were generated as previously described (49–51) and consisted of 453 formalin-fixed, paraffin-embedded RCC tissues along with corresponding normal kidney tissue not adjacent to the tumor. The study was approved by the Ethical Committee of the University of Erlangen-Nuremberg (Germany) and conducted according to the principles expressed in the Declaration of Helsinki. The specimens were collected between 1998 and 2011. All specimens were reevaluated by two experienced pathologists (A. Hartmann, V. Spath) including the classification of the RCC subtype and the WHO grade according to the 2004 World Health Organization (WHO) classification. A summary of patient and tumor characteristics can be found in Table 2.

IHC of 5 μm sections cut from the TMA tissue blocks was performed using the HLAG-specific mAb “4H84” (Abcam, Cat. no. ab52455) at a 1:50 dilution generated with the Antibody Diluent (Dako, Cat. no. S080981–2). In parallel, immune cell infiltrates were monitored using antibodies directed against CD3 (mAb SP7, Thermo Scientific, Cat. no. MA1–90582), CD4 (mAb 1F6, Leica, Cat. no. CD4–1F6-L-CE), CD8 (mAb C8/144B, Dako, Cat. no. IS62330–2), CD56 (mAb MRQ-42, Cell Marque Corporation, Cat. no. 156R–97), CD25 (mAb 4C9, Cell Marque Corporation, Cat. no. 125M–17), CD69 (mAb CH11, Leica, Cat. no. NCL−CD69) and FoxP3 (mAb 236A/E7, Abcam, Cat. no. ab20034), respectively. For visualization, the EnvisionTM detection system (Dako) including a horseradish peroxidase (HRP)-linked anti-mouse secondary antibody and
| Primer          | Application                | Sequence (5′ → 3′)                                      | Condition | Reference |
|-----------------|----------------------------|--------------------------------------------------------|-----------|-----------|
| 141-RT-Rkt      | Stem-loop primer           | GTCGTATCCAGTGCAGGGTCCGAAGGTATTCGCACTGGATACGACCCATCT   | 42°C      |           |
| 141 PCR fw      | qRT-PCR/PCR                | GCCCTAAGCTCGAAGGTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| 148a-RT-Rkt     | Stem-loop primer           | GTCGTATCCAGTGCAGGGTCCGAAGGTATTCGCACTGGATACGACCCATCT   | 42°C      |           |
| 148a PCR fw     | qRT-PCR/PCR                | GCCCTAAGCTCGAAGGTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| 152-RT-Rkt      | Stem-loop primer           | GTCGTATCCAGTGCAGGGTCCGAAGGTATTCGCACTGGATACGACCCATCT   | 42°C      |           |
| 152 PCR fw      | qRT-PCR/PCR                | GCCCTAAGCTCGAAGGTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| 148b-RT-Rkt     | Stem-loop primer           | GTCGTATCCAGTGCAGGGTCCGAAGGTATTCGCACTGGATACGACCCATCT   | 42°C      |           |
| 148b PCR fw     | qRT-PCR/PCR                | GCCCTAAGCTCGAAGGTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| 133ART-Rkt      | Stem-loop primer           | GTCGTATCCAGTGCAGGGTCCGAAGGTATTCGCACTGGATACGACCCATCT   | 42°C      |           |
| 133A PCR fw     | qRT-PCR/PCR                | GCCCTAAGCTCGAAGGTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| 541-RT-Rkt      | Stem-loop primer           | GTCGTATCCAGTGCAGGGTCCGAAGGTATTCGCACTGGATACGACCCATCT   | 42°C      |           |
| 541 PCR fw      | qRT-PCR/PCR                | GCCCTAAGCTCGAAGGTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| stem loop reverse primer | qRT-PCR/PCR                | GTGCACTGGATACGACACAAAGGTATTCGCACTGGATACGACCCATCT   | 42°C      |           |
| HLAGEX2se       | PCR                        | GTGCACTGGATACGACACAAAGGTATTCGCACTGGATACGACCCATCT   | 60°C      |           |
| SeeHLAGEX2      | Sequencing                 | AGGATGGGGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| SeeHLAGEX3      | Sequencing                 | AGGATGGGGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| SeqHLAGEX4      | Sequencing                 | AGGATGGGGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| 2.15 5′ UTR HLA-G fw cloned primer AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| 2.15 3′ UTR HLA-G rev cloned primer AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| Klonmir-152fw   | Cloning                    | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| Klonmir-152rev  | Cloning                    | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| Klonmir-541fw   | Cloning                    | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| Klonmir-541rev  | Cloning                    | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| HLAGmiTrap      | Cloning                    | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| 3′-UTR HLA-G fw Cloning | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| 3′-UTR HLA-G rev Cloning | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| 3′-UTR β-Actin fw Cloning | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| 3′-UTR β-Actin rev Cloning | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| mutPrimerfw     | Site-directed mutagenesis  | ACTTACCTCGAGGACTTACCTCGAGGACTTCGAGGTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| mutPrimerrev    | Site-directed mutagenesis  | ACTTACCTCGAGGACTTACCTCGAGGACTTCGAGGTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| HLAGrtpcrDBfw   | qRT-PCR/PCR                | GTGCACTGGATACGACACAAAGGTATTCGCACTGGATACGACCCATCT   | 60°C      |           |
| HLAGrtpcrDBrev  | qRT-PCR/PCR                | GTGCACTGGATACGACACAAAGGTATTCGCACTGGATACGACCCATCT   | 60°C      |           |
| Forward GAPDH   | qRT-PCR/PCR                | GTGCACTGGATACGACACAAAGGTATTCGCACTGGATACGACCCATCT   | 60°C      |           |
| Reverse GAPDH   | qRT-PCR/PCR                | GTGCACTGGATACGACACAAAGGTATTCGCACTGGATACGACCCATCT   | 60°C      |           |
| Klonmir-152fw   | Cloning                    | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| Klonmir-152rev  | Cloning                    | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| Klonmir-541fw   | Cloning                    | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| Klonmir-541rev  | Cloning                    | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| HLAGmiTrap      | Cloning                    | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| newmiTRAPrev    | Cloning                    | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| Klonmir-152fw   | Cloning                    | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| Klonmir-152rev  | Cloning                    | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| Klonmir-541fw   | Cloning                    | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| Klonmir-541rev  | Cloning                    | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| HLAGmiTrap      | Cloning                    | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| newmiTRAPrev    | Cloning                    | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| 152igo2        | Hybridization/cloning      | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| 152igo3        | Hybridization/cloning      | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| 152igo4        | Hybridization/cloning      | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
| 152igo5        | Hybridization/cloning      | AAGAGCTAGCTTGCTGTTGACATGCGAGGTTGGTATTCTCTCTCGGACAGAAG  | 60°C      |           |
the 3,3'-diaminobenzidine (DAB') substrate chromogen (Dako) was employed.

Immune cell infiltrates were quantified as mean of the absolute number of positive cells in four high-power fields (magnification x400). HLA-G expression was quantified using an immunoreactive score (IRS) by multiplying the percentage of positive tumor cells by the respective staining intensity (0: negative, 1: weak, 2: moderate, 3: strong staining). The digit in the brackets means the percentage of positively stained cells on the compound.

Cell lines and tissue culture

The HLA-G negative human embryonal kidney cell line HEK293T, the HLA-G positive choriocarcinoma cell line JEG-3, the murine fibroblast cell line NIH/3T3 (all purchased from the American Type Culture Collection (ATCC® CRL-3216™ and ATCC® HTB-36™, ATCC, Manassas, USA)) and a set of five established RCC cell lines derived from patients with RCC (MZ1257RC, MZ1790RC, MZ1795RC, MZ22733RC and MZ2905RC) have been employed. With the exception of JEG-3 cells, which were maintained in RPMI 1640 (Invitrogen), cell lines were cultured in Dulbecco's modified Eagles medium (DMEM) (Invitrogen) supplemented with 1% sodium pyruvate, 2 mM L-glutamine and 1% penicillin/streptomycin (V/V; PAA).

Purified lymphokine-activated killer (LAK) and NK cells derived from peripheral blood samples of healthy volunteers were generated as previously described.15 The identity and purity of NK (96.6%) and LAK cells (92.3%) were confirmed by flow cytometry. The purified NK and LAK cells were cultured in X-vivo 15 medium (Lonza), supplemented with 10% (V/V) fetal bovine serum (FCS) (PAA), 2 mM L-glutamine (Lonza) and 1% penicillin/streptomycin (V/V; PAA).

Protein extraction and western blot analysis

Protein extraction and Western blot analyses were performed as recently described.51 HLA-G protein was detected by using the HLA-G-specific mAb MEM-G/4 (Exbio, Cat. no. Eleven–ER0051, Cat. no. ER0561) replacing the luciferase (luc).

miR enrichment assay (miTRAP)

To enrich HLA-G-specific miRs, the recently published miTRAP method was employed.34 Briefly, the complete 3'-UTR of HLA-G was cloned upstream of four MS2 loops, in vitro transcribed with Ribobprobe (Promega, Cat. no. P1440) and used for the enrichment of HLA-G-specific miRs from cell lysates of the RCC cell line MZ2905RC (HLA-G mRNA+/ protein-). By application of different amounts of fusion protein consisting of the MS2 loop and maltose binding protein domain, 500 pmol of the in vitro-transcribed RNAs (HLA-G 3'-UTR and as a mock control a sequence encoding only the four MS2 loops) were loaded onto amyllose beads (NEB, Cat. no. E8021S). After washing and blocking steps with yeast tRNA (Promega) and BSA...
were significant with a sample assuming unequal variances have been selected. The data p \textless \text{log rank test (SPSS). Differences were regarded as significant at miR expression and disease-specific survival were calculated by Rho test was applied (SPSS). Associations of staining results, in order the significance of associations between staining results, were calculated using IBM SPSS Statistics 21 (IBM Corporation). For test-if lower than 0.005 with two stars).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Material
Supplemental data for this article can be accessed on the publisher’s website.

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