HLA-G 14bp ins/del Polymorphism, Plasma Level of Soluble HLA-G and Association with IL-6/IL-10 Ratio and Survival of Glioma Patients

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Research article

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Abstract: HLA-G is an immune checkpoint molecule with immunosuppressive and anti-inflammatory activities, and its expression and level of its soluble form (sHLA-G) may play an important role in tumor prognosis. The HLA-G 14 bp ins/del polymorphism and the plasma level of soluble HLA-G (sHLA-G) were investigated by polymerase chain reaction and ELISA, respectively, in 59 glioma patients. Significantly higher proportion of glioma patients had the 14 nt insert in both homozygous and heterozygous states compared to control group. Glioma patients had also higher plasma levels of sHLA-G. Patients with methylated MGMT promoter had lower levels of sHLA-G than those with unmethylated MGMT promoter. Level of sHLA-G negatively correlated with overall survival of patients. Glioblastoma patients who survived more than one year after diagnosis had lower levels of sHLA-G than those surviving less than one year. Patients with sHLA-G level below the cut off value 40 U/mL survived significantly longer than patients with sHLA-G above 40 U/mL. The levels of sHLA-G also negatively correlated with the level of IL-6 (P=0.0004) and positively with IL-10/IL-6 (P=0.046). Conclusion: The presence of 14 nt insert in both homozygous and heterozygous states of the HLA-G 14 bp ins/del polymorphism is more frequent in glioma patients and the elevated plasma levels of sHLA-G are negatively associated with their survival.

Key words: glioma, HLA-G, IL-6, polymorphism, prognosis, survival

1. Introduction

The HLA (human leukocyte antigen) system plays a key role in development of immune tolerance and has impact on both innate and adaptive immune response. HLA-G is one of the non-classical HLA class I molecules of the major histocompatibility complex with well-characterized immunomodulatory activities [1]. Contrary to the classical HLA antigens, HLA-G is characterized by a low polymorphism and high tolerogenic functions. It is coded by gene located on the short arm of the 6th chromosome (6p21.3), and encompasses at least four membrane-bound (mHLA-G: HLA-G1, HLA-G2, HLA-G3, and HLA-G4) and three soluble (sHLA-G: HLA-G5, HLA-G6, and HLA-G7) isoforms resulting from alternative splicing of its primary mRNA [2–5]. HLA-G gene consists of 8 exons; however, exon 8 remains untranslated due to the presence of a stop codon in exon 7. The non-translated region of exon 8 is termed “3′untranslated region (3’UTR)” [6]. Eighteen SNPs, a 14 bp insertion/deletion, and 44 haplotypes have been identified in the 3’UTR region [1], which are known to

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influence the translation of HLA-G proteins through either a reduced transcription, mRNA stability, or aberrant alternative splicing.

Under physiological conditions, HLA-G expression is highly tissue-restricted. It modulates the immune system activity in tissues where physiological immune-tolerance is necessary – e.g. in the cytotrophoblast at maternal – fetal interface [7] and in adults, in immune-privileged organs, including the cornea, thymus, pancreatic islets, endothelial cell precursors, and erythroblasts [2]. HLA-G plays a major role by protecting both the semi-allogenic tissues of the fetus from maternal immune system and immune privileged organs by creating a tolerogenic microenvironment

HLA-G is a tolerogenic molecule, that inhibiting effector cells, or generating regulatory subtypes broadly regulates the immune system – both innate and adaptive immune responses and inflammation [8]. It is involved in maintaining tolerance in autoimmune, inflammatory diseases [9–11], and after transplantation [12–15], and its immunosuppressive and anti-inflammatory functions have been well recognised [16]. HLA-G potentiates also the immune escape in cancer and infectious diseases [17,18].

Many studies previously described the neo-expression of HLA-G in different types of cancer cells, and its correlation with histological grade [19] bad prognosis [20,21], tumor metastasis and poor survival [22]. HLA-G plays a great role in creating a tolerogenic tumor microenvironment.

For proper anti-tumor immunity both a well-functioning innate and adaptive Th1 immunity are required. However, tumor cells have developed various direct and indirect mechanisms impairing the functions of immune cells in tumor microenvironment for their survival. Aberrant induction of HLA-G expression in malignant cells represents one of the key factors that contributes to tumor immune escape and progression. HLA-G with its immunosuppressive functions hampers the anti-tumor immunity and potentiate the cancer growth and its metastatic process. It plays a great role in all three phases of immuneoediting – 1. Elimination, 2. Equilibrium, and 3. Escape (three E) [23,24].

HLA-G exerts its immunosuppressive activity by various mechanisms, e g. 1. through binding to inhibitory receptors on immune cells. These inhibitory receptors like immunoglobulin-like transcript (ILT) 4, are negative regulator of immune response not only in allograft rejection, autoimmunity and infectious diseases, but also in tumor development [25]. To date, several inhibitory receptors for HLA-G have been identified, such as CD85j/immunoglobulin-like transcript 2 (ILT2), CD85d/ILT4, and CD158d/killer cell

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immunoglobulin-like receptor 2DL4 (KIR2DL4). Moreover, CD8 and CD160 have also been reported to strongly bind HLA-G [26]. HLA-G even up-regulates ILT2, ILT3, ILT4, KIR2DL4 and LILRB1 inhibitory receptors in antigen presenting cells, NK cells, and T cells [25,27]. Binding of both membrane bound or soluble HLA-G to inhibitory receptors on immune cells, HLA-G directly inhibits the functions of these effector cells leading to immune suppression, e.g. NK cells [25, 28–29] cytotoxic T- lymphocytes (CTL) [30], T and B cells [31,32] – monocytes, macrophages, neutrophils, myeloid derived stem cells (MDSC), $CD8^+PD\text{-}1^-ILT2^+$ intra-tumoral T cells [33] and dendritic cells (DC) [34,35].

Cai et al. (2019) in their study showed the co-expression of ILT4 and HLA-G in tissues of human primary colorectal cancer (CRC). They revealed, that the co-expression of ILT4 and HLA-G in tissues of human primary CRC and their mutual interaction promotes the progression of human colorectal cancer. The association of HLA-G with older age, advanced stage, regional lymph node involvement and poor overall survival time was observed [36].

Binding of sHLA-G to CD8 molecule of cytotoxic T cells and NK cells induces their apoptosis [37,38]. Ajith et al. (2019) demonstrated a novel mechanism by which HLA-G dimer inhibits activation and cytotoxic capabilities of human $CD8^+$ T cells. This mechanism implicated the down-regulation of granzyme B expression and the essential involvement of the inhibitory receptor LILRB1 [39]. The same mechanism might have a negative anti-tumor effect.

As an indirect effect, HLA-G inhibits the proliferation of allo-specific T cells, maturation and function of B cells and may lead to generation of HLA-G expressing tolerogenic DC-10 [32,40,41]. $HLA\text{-}G^+$ antigen presenting cells can induce immunosuppressive $CD4^+$ T cells and, in the case of DC-10, HLA-G mediates the generation of type 1 regulatory T cells [41,42].

2. The second mechanism of immunosuppressive activity of HLA-G is done by transfering this immunosuppressive molecule from tumor or immune cells to other immune cells through trogocytosis, exosomes and tunnelling nanotubes. This way, $HLA\text{-}G^{acq+}$ $CD4^+$ T cells, $HLA\text{-}G^{acq+}$ $CD8^+$ T cells, $HLA\text{-}G^{acq+}$ NK cells and $HLA\text{-}G^{acq+}$ $CD14^+$ monocytes with immunosuppressive and regulatory activity are formed, further amplifying the tolerogenic effects of HLA-G in tumor immune escape which leads to immune evasion, therapy resistance, disease progression and poor clinical outcome [43]. Induction of these $HLA\text{-}G^+$ tolerogenic cells, including DC-10 leads to long-lasting immune-regulatory activities [12,22].

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Due to its immunosuppressive activity, the negative role of HLA-G has been found to be related to the development of the tumor process. HLA-G overexpression has been associated with the development of several solid tumors and contributed to their immune evasion [44]. Its expression has also been correlated with poor clinical outcome of cancer patients [44,45].

Many studies have claimed HLA-G as a new immune checkpoint in cancer [46]. Recent medical investigations suggest that HLA-G can be used as a biomarker in the diagnosis, treatment, and prognosis of different neoplasms. However, the potential diagnostic value of sHLA-G with other tumor markers in gliomas has not been explored yet.

As the expression of HLA-G is finely tuned by genetic variations (polymorphisms) in the non-coding region of the locus, besides plasma levels of soluble HLA-G in glioma patients, we investigated also the gene polymorphism of this molecule in glioma patients. An important polymorphism is the presence of the 14 nt sequence (insert, rs16375) in the 3 'non-transcribed region of the HLA-G gene, as it was found to negatively affect the stability of mRNA and hence total serum soluble HLA-G (sHLA-G) levels [47–49]. We were also interested in whether and how the plasma levels of sHLA-G are related to the methylation of the MGMT promoter, which affects the efficacy of treating glioma patients [50]. We also evaluated a possible correlation of sHLA-G with the level of immunoregulatory and anti-inflammatory cytokine IL-10, pro-inflammatory cytokine IL-6 and their ratio (IL-10 / IL-6), and the association of sHLA-G with survival of glioma patients. Indeed, in addition to good cell mediated immunity, it is precisely the inflammation, that plays an important role in the pathogenesis of gliomas [51].

2. Subjects and Methods

2.1. Study Groups

59 patients (25 women, 34 men) with gliomas were enrolled in our study. Out of them, 49 had primary gliomas and 10 recurrent gliomas. The mean age of the patients at the time of diagnosis was 53.36 ± 15.17 years. 19 patients had glioma grade II, 11 of grade III and 29 glioma grade IV (Table 1). The control group compromises of 159 healthy subjects without cancer diagnosis (80 women, 79 men with the mean age 40.91 ± 11.93 years).

TABLE 1. Characteristics of the study group of glioma patients

Parameter	Brain gliomas

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(Mean ±SD)	N = 59
Age at diagnosis	53.36 ± 15.17
Sex (women/men)	25/34
Grade of gliomas	
Grade II	19
Grade III	11
Grade IV	29
Primary diagnosis of glioma	49
Relapse	10

N – number of patients; SD – standard deviation

2.2. Procedures and Sample Processing

10 ml of blood was collected from glioma patients in a tube with EDTA (ethylenediaminetetraacetic acid) and 5 ml of blood in a tube without anticoagulant in the morning of surgery at the Department of Neurosurgery of the Faculty of Medicine, Comenius University and University Hospital in Bratislava. Approximately 1.5 ml of blood was collected from an EDTA tube and immediately transferred to the Laboratory of Immunology, Medirex, Ltd. The remaining samples were immediately centrifuged in our laboratory and the plasma (from EDTA tube) or serum from the tube without anticoagulant were withdrawn, aliquoted into micro tubes and stored in a deep-freezer box at -80° C until examination. DNA was isolated from the remainder blood (after the withdrawal of the plasma) by the salting out procedure [52] and stored at -20° C. Later, this DNA was used to examine the HLA-G 14 bp insertion/deletion polymorphism in the 3' UTR (rs16375). The study was conducted in accordance with the Declaration of Helsinki, approved by the Ethical Committee of the Faculty of Medicine, Comenius University and University Hospital in Bratislava (Project identification code: 17/2015). Each patient received a written informed consent. The patients came from the Department of Neurosurgery of the Faculty of Medicine, Comenius University and the University Hospital in Bratislava. The histopathological diagnosis and grade of malignancy was investigated at Cytopathos, Ltd. and Alpha Medical., Ltd. in Bratislava.

HLA-G 14 bp ins/del polymorphism was investigated by polymerase chain reaction (PCR) as described by Hviid et al. (2002) [48]. Briefly, DNA was amplified by forward primer 5′GTGATGGGCTGTTTAAAGTGTCACC-3′ and reverse primer 5′GGAAGGAATGCAGTTCAGCATGA-3′ using a PCR cycler (Biometra). Reaction mixture with a total volume of 25 µl contained 50 ng of template DNA, 0.2 mM of each dNTP

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(Thermo Fisher Scientifi c), 1 unit of Taq DNA polymerase (Thermo Fisher Scientifi c), 1.5 mmol MgCl2 (Thermo Fisher Scientifi c) and 10 pmol of each specific primer. PCR conditions were 95 °C for 3 min, followed by 30 cycles (denaturation at 95 °C for 1 min, annealing at 64 °C for 1 min and elongation at 72 °C for 1 min) and final elongation at 72 °C for 10 min. The PCR products were run in 3% agarose gel for 20 min and then visualized under UV-light. Fragment size was confirmed using the 100 bp DNA ladder (SBS). PCR fragments of 224 bp (14 bp insertion) and PCR fragments of 210 bp (14 bp deletion) were identified.

The plasma level of soluble HLA-G (sHLA-G) was determined by sandwich ELISA according to manufacturer's recommended procedure (human sHLA-G ELISA kit; Exbio, BioVendor, Czech Republic). Concentrations of anti-inflammatory cytokine IL-10 (human IL-10 Elisa kit; Wuhan Fine Biotech Co., Ltd., China) and pro-inflammatory cytokine IL-6 in plasma of glioma patients (human IL-6 Elisa kit; Wuhan Fine Biotech Co., Ltd., China) were also determined by sandwich ELISA method. The survival time was calculated from the time of diagnosis until April 2019 or the time of death. Patients were monitored from 1 of December 2015 to 30 of April 2019.

2.3. Statistical analysis

Statistical significance of differences in allele and genotype frequencies between two studied groups (gliomas vs. controls) was evaluated by the standard chi square test using the InStat statistical software (GraphPad Software, Inc., San Diego, USA). The odds ratios (OR) and 95% confidence intervals (95% CI) were calculated as well. Finally, multivariate logistic regression analysis adjusted for sex and age as possible influencing factors was performed by the SNP stats web software available at https://snapstat.net/snpstats/ [53].

For statistical analysis we used programs InStat and SAS. We used Student´s t-test, Mann–Whitney test, the Cox proportional hazard analysis, Kaplan-Meier survival analysis, and Log Rank test. The Spearman correlation has also been tested. The results were expressed as the median and interquartile range (IQR), mean ± standard deviation (SD), and hazard ratio (HR). P value < 0.05 was considered to indicate the statistical significance.

3. Results

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3.1. Comparison of HLA-G 14 bp ins/del Allele and Genotype Frequencies Between Glioma Patients and Control Group of Healthy Subjects

In a group of 59 patients with brain gliomas and 159 controls, the presence of 14 nt insert (14ins) in the 3'UTR region of HLA-G by PCR were analyzed. The differences in the frequencies of the HLA-G alleles and genotypes carrying the 14 nt insert between the examined groups were calculated using the chi-square test. The group of glioma patients comprised significantly higher proportion of individuals carrying the 14 nt insert in both homozygous and heterozygous forms (14ins/ins and 14del/ins) compared to the control group of healthy subjects (79.66% vs 65.41%; P = 0.03; Table 2). After adjustment for age and sex, no statistically significant association of HLA-G 14bp ins/del variants with gliomas was found.

TABLE 2. Allele and genotype frequencies of HLA-G 14 bp ins/del polymorphism in glioma patients and healthy controls

Allele/ genotype	Brain gliomas (N = 59)	Controls (N = 159)	Univariant analysis		Multivariante analysis	
			P	OR (95% CI)	P	OR (95% CI)
-14	58 (49.15%)	191 (60.06%)				
+14	60 (50.85%)	127 (39.94%)	0.05	1.56 (1.02 - 2.38)	-	-
-14/-14	12 (20.34%)	55 (34.59%)		1.00		1.00
-14/+14	34 (57.63%)	81 (50.94%)	0.09	1.92 (0.92-4.04)	0.35	1.64 (0.72-3.72)
+14/+14	13 (22.03%)	23 (14.47%)		2.59 (1.03-6.52)		2.00 (0.71-5.65)
-14/-14	12 (20.34%)	55 (34.59%)		1.00		1.00
-14/+14 and +14/+14	47 (79.66%)	104 (65.41%)	**0.03**	2.07 (1.02-4.23)	0.17	1.72 (0.78-3.78)
-14/-14 and -14/+14	46 (77.97%)	136 (85.53%)		1.00		1.00
+14/+14	13 (22.03%)	23 (14.47%)	0.19	1.67 (0.78-3.57)	0.43	1.43 (0.60-3.40)
-14/-14 and +14/+14	25 (42.37%)	78 (49.06%)		1.00		1.00

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-14/+14	34 (57.63%)	81 (50.94%)	0.38	1.31 (0.72-2.39)	0.54	1.24 (0.63-2.44)

Allele and genotype frequencies are presented as absolute numbers with percentages in parentheses. Univariate analysis is based on $\chi 2$ test. Multivariate analysis is adjusted by sex and age. OR - odds ratio; CI - confidence interval, N – number, +14bp – insertion of 14 bp sequence, -14bp – deletion of 14 bp sequence

3.2. Comparison of Plasma Levels of sHLA-G in Glioma Patients and Healthy Controls

Next, we analysed the level of immunosuppressive molecule sHLA-G in a group of patients with gliomas and healthy controls. We found a significantly higher plasma levels of sHLA-G in glioma patients compared to the healthy control population (P=0.048; Table 3).

TABLE 3. The plasma level of sHLA-G in glioma patients and healthy controls

	Patients (N=59)	Healthy controls (N=43)	P (Student´s T-test)
sHLA-G (U/ml) (Mean ± SD)	42.17 ± 38.50	23.06 ± 9.53	0.048

sHLA-G – soluble HLA-G, SD – standard deviation, P ≤ 0.05 is statistically significant

We were also interested in whether sHLA-G levels differ in patients with gliomas at different stages of the disease. However, no statistically significant differences in sHLA-G levels among different gliomas grades were found (Table 4).

TABLE 4. Comparison of plasma levels of sHLA-G in different stages of glioma in primary diagnosis (N = 49)

	Grade 2 N=13	Grade 3 N=9	Grade 4 N=27	P (2 *vs* 3)	P (2 *vs* 4)	P (3 *vs* 4)
sHLA-G (U/ml) (Mean ± SD)	39.19 ± 40.96	32.05 ± 13.69	43.23 ± 39.42	0.45	0.22	0.70

sHLA-G – soluble HLA-G, SD – standard deviation, P ≤ 0.05 is statistically significant

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3.3. The Effect of HLA-G 14 bp ins/del Polymorphism on sHLA-G Plasma Levels in Glioma Patients

In the group of patients with gliomas, we compared the level of sHLA-G with HLA-G 14 bp ins/del variants to determine if the investigated polymorphism affects the plasma sHLA-G level. The level of sHLA-G between individuals with different variants of the HLA-G bp 14 ins/del genotypes was not statistically different (P = 0.395 - 0.957; Table 5).

TABLE 5. Association between HLA-G 14 bp ins/del polymorphism and the level of sHLA-G in glioma patients (N = 59)

Allele/genotyp	sHLA-G (U/ml) Mean ± SD	P
A. -14 (N = 58)	44.586 ± 38.34	
B. +14 (N = 60)	37.942 ± 29.81	0.499 (A *vs*. B)
C. -14/-14 (N = 12)	45.76 ± 36.17	0.395 (C *vs*. D)
D. -14/+14 (N = 34)	41.06 ± 35.77	0.468 (D *vs*. E)
E. +14/+14 (N = 13)	48.56 ± 39.06	0.957 (C *vs*. E)

N – number of patients; sHLA-G – soluble HLA-G, SD – standard deviation; P ≤ 0.05 is statistically significant

3.4. Analysis of the Association of Plasma Levels of sHLA-G with a O^6-Methylguanine-DNA Methyl-Transferase (MGMT) Promoter Methylation Status in Glioma Patients

Comparing plasma levels of sHLA-G in a group of patients with methylated and unmethylated MGMT promoter, patients with methylated MGMT promoter were shown to have lower plasma levels of the immunosuppressive molecule sHLA-G than those with unmethylated MGMT promoter. This suggests that patients with methylated MGMT promoter

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who respond better to treatment have significantly lower plasma concentrations of sHLA-G (mean: 29.51 U / ml *vs* 54.30 U / ml; P = 0.03; Table 6).

TABLE 6. Plasma levels of sHLA-G in glioma patients with different MGMT promoter methylation status

MGMT promoter	Patients (N=32)	sHLA-G (U/ml) (Mean ± SD)	P
methylated	17	29.51 ± 23.50	**0.03**
unmethylated	15	54.30 ± 43.12	

N – number of patients; sHLA-G – soluble HLA-G, SD – standard deviation; P ≤ 0.05 is statistically significant

3.5. Association of Plasma Levels of Immunosuppressive Molecule sHLA-G with the Levels of Pro-inflammatory Cytokine IL-6, Anti-inflammatory Cytokine IL-10, and IL-10/IL-6 Ratio in Glioma Patients

Since inflammation plays an important role in the pathogenesis of gliomas, we wondered whether the level of sHLA-G in plasma affects the concentrations of the selected pro- and anti-inflammatory cytokine. The results showed that the levels of sHLA-G negatively highly significantly correlated with the pro-inflammatory cytokine IL-6 concentration (Table 7, P = 0.0004). However, we did not find correlation with the level of the anti-inflammatory cytokine IL-10, but found a positive correlation with the ratio of IL-10/IL-6 levels in the plasma of glioma patients (P = 0.046; Table 7).

TABLE 7. Correlation of plasma levels of sHLA-G with IL-6, IL-10 levels and IL-10/IL-6 in glioma patients

Patients (N=32)	Correlation of sHLA-G with IL-6	Correlation of sHLA-G with IL-10	Correlation of sHLA-G with IL-10/IL-6 ratio
Spearman r	-0.584	0.208	0.622

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95 % CI	-0.779 – - 0.286	-0.527 – 0.163	-0.0034 – 0.633
P	**0.0004**	**0.26**	**0.046**

N – number of patients, sHLA-G – soluble HLA-G; CI - confidence interval, P ≤ 0.05 is statistically significant

3.6. Association of Plasma Levels of sHLA-G with Survival Time in Grade II and IV Glioma Patients

When we performed Cox hazard proportional analyses, sHLA-G was proven to have influence on overall survival time in grade II (*HR*=1.023; *P*=0.0088) and IV (*HR*=1.004; *P*=0.0399) glioma patients (Table 8). Survival time is reduced with plasma sHLA-G increase. In grade III glioma patients this association was not observed.

TABLE 8. Cox proportional hazard analysis of sHLA-G and survival time in grade II and IV glioma patients

Parameter		Parameter estimate	Standard error	Chi-square	P	Hazard ratio
sHLA-G in G. II (N = 19)	Survival time (months)	0.02234	0.00852	6.8696	0.0088	1.023
sHLA-G in G. IV (N = 29)	Survival time (months)	0.00435	0.00212	4.2222	0.0399	1.004

3.7. Correlation Between Plasma Level of sHLA with Overall Survival in Whole Group of Glioma Patients and in the Subgroup of Glioblastoma Patients

The plasma level of sHLA-G negatively correlated with overall survival in whole group of glioma patients (Spearman r = -0.25, P = 0.05, 95% CI: -0.4825 - 0.0085). Glioblastoma patients (G IV) who survived more than one year after diagnosis had significantly lower plasma values of sHLA-G than patients who survived less than one year (median 21.5 U/ml *vs.* 46.74 U/ml, P = 0.02) (Table 9). We also observed significant difference in overall survival of G IV patients when we compared patients with sHLA-G above and below cut off of sHLA-G 40 U/ml. Patients with the level of sHLA-G below 40

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U/ml survived significantly longer than patients with sHLA-G above 40 U/ml (P=0.038, Figure 1, Table 10). In G. II the difference did not reach the statistical significance (P = 0.06) and in G. III was not significant at all, probably due to low number of patients.

TABLE 9. Comparison of sHLA-G in patients with G. IV who survived more than one year and less than one year (non-parametric test)

sHLA-G (U/ml)	Pts. surviving <1 year N=21	Pts. surviving >1 year N=8
Median	46.74	21.50
IQR	74.80	12.60
95% CI	30.02-119.10	14.59-30.17
P	**0.02**	

N – number, G – grade, Pts. – patients, CI – confidence interval, P < 0.05 is statistically significant

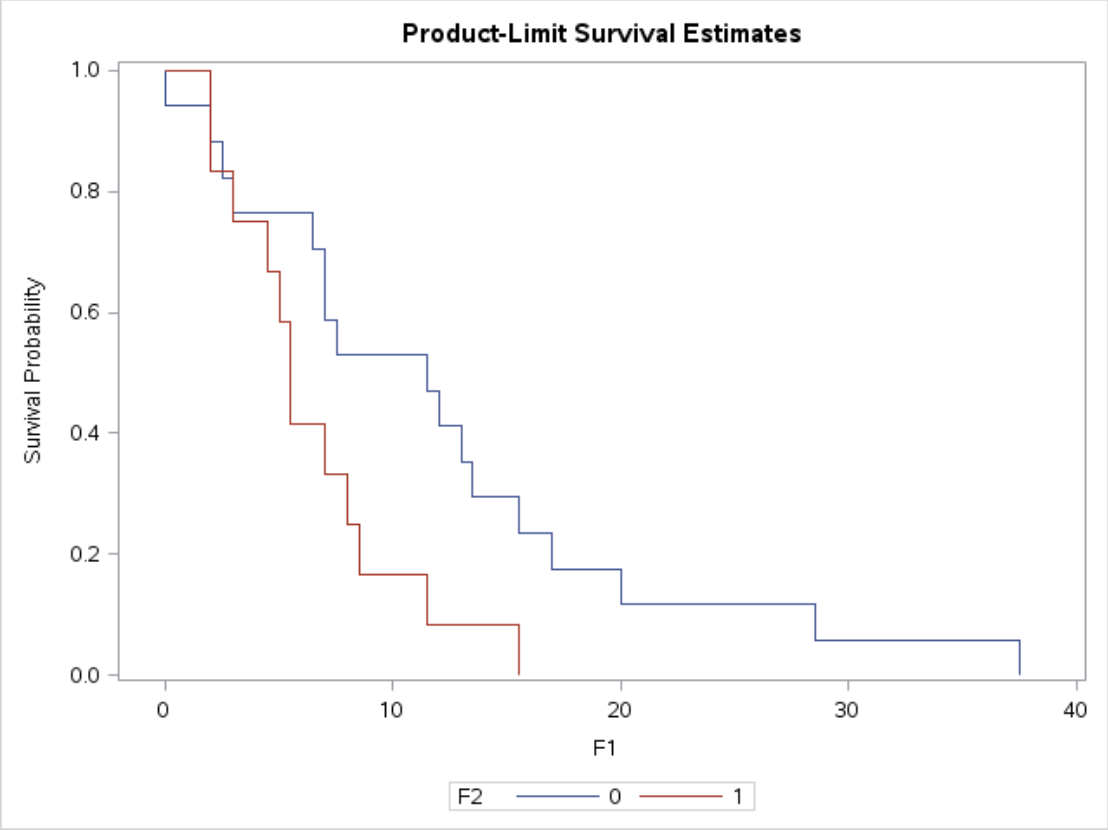

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FIGURE 1. Kaplan-Meier survival curves of grade IV according to sHLA-G, 0 – patients with sHLA-G < 40 (N = 17), 1 - patients with sHLA-G > 40 (N = 12), X axis – survival time in months, Y axis – survival probability (Test: Log rank P = 0.038)

TABLE 10. Comparison of mean survival time in patients with sHLA-G above and below 40 U/mL in G. IV

	sHLA-G < 40 U/mL (N=17)	sHLA-G > 40 U/mL (N=12)
Mean survival time ± SD (months)	12 ± 9.82	6.5 ± 3.97
95% CI	6.95 – 17.05	3.98 – 9.02

G – grade, CI – confidence interval, P < 0.05 is statistically significant

4. Discussion

From immunological point of view, the development and progression of tumors is influenced by two main immune factors: decreased Th1 immunity and increased inflammatory process or presence of at least of chronic low-grade inflammation. Many studies have claimed HLA-G as a new immune checkpoint in cancer [46]. Emerging evidences indicate that tissue expression of HLA-G, as well as HLA-G-expressing regulatory cells, and the level of soluble HLA-G may play an important role in dictating the outcome of the anti-tumor immune response [54].

HLA-G expression in tumor lesions was first demonstrated in melanoma [55] and later, its expression has been correlated with poor clinical outcome of various cancer patients [44,45, 56–59]. In recent years there have been some reports that point to its importance in patients with gliomas [45].

In our previous study analysing HLA-G 5'URR, we found genetic association of haploblock consisting of −762T, −716G, −689G, −666T and −633A allele followed by −486C and −201A alleles with susceptibility to develop gliomas for the first time. In the grade IV glioma patients, we also observed that haploblock carriers of −762CT, −716TG, −689AG,

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−666GT, −633GA, −486AC, −477GC, −201GA followed by −369AC carriers tend to have lower age at onset as compared to other genotype carriers. However, no correlation of HLA-G 5'URR variants with sHLA-G plasma level was found [60]. There is only one study of Magalhaes et al. (2020) analysing the association of the 14 bp ins/del polymorphisms of the HLA-G 3' UTR and its relation with plasma sHLA-G level in glioma patients published in april 2020 [61]. As it is the first study concerning HLA-G 14 bp ins/del polymorphism in gliomas, our study is the second one.

We analyzed the presence of 14 nt insert in the 3'UTR region of HLA-G and found a significantly higher proportion of glioma patients carrying the 14 nt insert in both homozygous and heterozygous states (14ins/ins and 14del/ins) compared to the control group of healthy subjects (P = 0.03). Lau et al. (2011) reported no association of 14-bp ins/del polymorphism with risk of childhood neuroblastoma and their analyses did not detect an association between common HLA-G polymorphisms and clinical outcome in patients treated for neuroblastoma [62]. The prevalence of the HLA-G genotype carrying the 14 nt insert was also found in patients with other solid tumors [63–66]. However, there is also some opposite evidence that the presence of the HLA-G 14 nt insert is associated with a reduced risk of developing malignancies [67].

Glioma patients in our study compared to healthy controls had also higher plasma levels of sHLA-G (P = 0.009), which is consistent with the results of other studies [68]. To determine if the investigated polymorphism affects the plasma level of sHLA-G in glioma patients, we compared the level of sHLA-G in patients with different variants of the HLA-G 14 ins/del genotypes, however, the differences were not statistically significant. It might be explained by the fact, that the levels of sHLA-G in glioma patients are influenced not only by HLA-G variants, but also by environmental factors, such as treatment, hormones, stress, and hypoxia [69,70]. However, Magalhaes et al. (2020) found an association of the of the heterozygous 14 bp ins/del and +3142 C/G genotypes of the HLA-G 3' UTR with higher HLA-G plasma levels in grade IV glioma patients when compared with controls [61].

Comparing plasma levels of sHLA-G in our group of patients with methylated and unmethylated MGMT (methylguanidine methyltransferase) promoter, patients with methylated MGMT promoter were shown to have lower plasma levels of the immunosuppressive molecule sHLA-G than those with unmethylated MGMT promoter. This suggests that patients with methylated MGMT promoter who respond better to treatment have significantly lower plasma concentrations of sHLA-G (P = 0.03). The explanation for this is

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the knowledge, that patients with a methylated MGMT promoter are known to respond better to treatment with alkylating cytostatics, e.g. Temozolomide. Temozolomide alkylates DNA bases and damages tumor cells. If this enzyme (MGMT) is functional in the patient, it corrects what temozolomide kills and thus worsens the patient's prognosis. Patients with a methylated MGMT promoter have a less functional MGMT enzyme and respond better to treatment with particular temozolomide [71]. We did not find any study concerning association of the level of sHLA-G with the methylation status of MGMT.

Further, we proved the influence of plasma sHLA-G on overall survival time in grade II (P=0.0088) and grade IV (P=0.0399) glioma patients. Survival time is reduced with plasma sHLA-G increase. We also analyzed the correlation between plasma level of sHLA-G with overall survival in whole group of glioma patients and in the subgroup of glioblastoma (G IV) patients. We found that the plasma level of sHLA-G negatively correlated with overall survival in whole group of glioma patients (P = 0.05). Glioblastoma patients who survived more than one year after diagnosis had significantly lower plasma values of sHLA-G than patients who survived less than one year (P = 0.02). We also observed a significant difference in overall survival of G IV patients when we compared patients with sHLA-G above and below the cut off of sHLA-G 40 U/mL (P = 0.038). Patients with the level of sHLA-G below 40 U/mL survived significantly longer time than patients with sHLA-G above 40 U/mL.

There exists consistent evidence in the literature that plasma levels of sHLA-G are higher in cancer patients than in healthy controls. This was proven in breast cancer, gastrointestinal tumors, lung and urogenital cancer [72-74]. Also, Kirana et al. (2017) found an association of higher sHLA-G level with worse prognosis in colorectal cancer [75,76].

In our study we observed higher plasma levels of sHLA-G in patients with gliomas than in healthy controls. We think, that soluble HLA-G could be released from tumors to help them escape from immune surveillance of the body. We suppose that increased level of immuno-suppressive sHLA-G in the peripheral blood inhibits the anti-tumor immunity, helps tumor growth, promotes faster progression and shorter overall survival. This hypothesis is supported by our finding, that patients with GBM, who survived less than one year had significantly higher values of sHLA-G.

Since inflammation plays an important role in the pathogenesis of gliomas, we wondered whether the level of sHLA-G in plasma affects the concentrations of the selected pro- and anti-inflammatory cytokine. The results showed that the levels of sHLA-G negatively highly significantly correlated with the concentration of pro-inflammatory cytokine

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IL-6 (P = 0.0004), thus likely contributing to suppressing its production. However, we did not find correlation with the level of the anti-inflammatory cytokine IL-10 (sHLA-G does not affect its formation), but found a positive correlation with the ratio of IL-10 / IL-6 levels in the plasma of glioma patients (Table 9; P = 0.046). We found a study, in which authors showed, that IL-10 increases the expression of HLA-G [77].

Taking into account both actual knowledge and our results we suppose that HLA-G appears as a promising relevant target for cancer immunotherapy.

5. Conclusions

Higher proportion of glioma patients had the 14 nt insert at the HLA-G 3'UTR in both homozygous and heterozygous states compared to the control group. Glioma patients had also higher plasma levels of sHLA-G. The level of this immunosuppressive and anti-inflammatory molecule was lower in patients with methylated MGMT promoter than those with unmethylated MGMT promoter. We proved the influence of plasma sHLA-G on overall survival time in grade II and IV glioma patients. Survival time reduced with plasma sHLA-G increase. The plasma level of sHLA-G negatively correlated with overall survival in whole group of all glioma patients. Glioblastoma patients who survived more than one year after diagnosis had significantly lower plasma values of sHLA-G than patients who survived less than one year. The overall survival of G IV patients with the level of sHLA-G below 40 U/mL was significantly longer than of patients with sHLA-G above 40 U/mL. The levels of sHLA-G negatively correlated with the concentration of pro-inflammatory cytokine IL-6 and positively with IL-10/IL-6 ratio in plasma of glioma patients. The HLA-G 14 bp ins/del polymorphism is associated with increased susceptibility to develop gliomas and the plasma level of sHLA-G have impact on survival of glioma patients.

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