The non-classical HLA molecule HLA-G has role in the pathogenesis of gliomas

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Background

Gliomas are brain tumors that have the third highest mortality and morbidity rates among cancers in human population. Gliomas begin in glial cells that surround nerve cells and help them function and are classified as grade I to IV according to histological features and genetic alterations, as defined by the WHO (1). Grade IV tumors, also known as glioblastoma multiforme (GBM) are associated with the worst prognosis (2). Despite the development of various therapeutic strategies, the prognosis of patients with malignant glioma remains poor. The markers of survival prognosis in patients with gliomas are still evaluated.
One of the potential biomarkers of diagnosis and prognosis in malignancies is Human leukocyte antigen G (HLA-G). The HLA-G is a non-classical HLA-class I molecule and is involved in the suppression of immune response. The HLA-G is mainly expressed in extravillous cytotrophoblast that helps to protect the fetus from maternal immune rejection (3). In tumors, HLA-G is involved in escape from immune cell recognition and subsequent tumor elimination. There are two forms of HLA-G – membrane bound and soluble (sHLA-G). Under physiological condition, HLA-G plays a great role in fetal tolerance and pregnancy. It has been proven that the expression of "embryonic" HLA groups (HLA-G, HLA-E, HLA-F) by tumor cells leads to a similar inhibition of the innate and adaptive immune system and by this way facilitate tumor immune escape (4).
The HLA-G gene is characterized by low polymorphism, namely, 69 HLA-G alleles, 19 HLA-G proteins, and 3 null alleles have been identified to date (5). Increased HLA-G expression can be associated with polymorphic sites in two noncoding regions: the upstream regulatory region (5′ URR) and untranslated region (3′UTR). At the 3′UTR, the 14 bp insertion/deletion, affecting mRNA stability, was associated with susceptibility to certain cancer types (6).
HLA-G 5′URR consist of the 1.4 kb upstream the ATG and shows at least 35 single nucleotide polymorphisms (SNPs) defining 68 haplotypes, of which 9 - 10 have been frequently observed in human populations (7). According to our knowledge, no study evaluated the association between HLA-G 5'URR polymorphisms and risk for glioma development so far. Thus, the aim of the study was to analyze the association between 16 polymorphic variants in HLA-G 5′URR region, sHLA-G level, and clinical variables in patients with gliomas.

Subjects and Methods

The study involved 59 patients with gliomas (34 males, 25 females) aged 24 - 77 years (mean age 54.70 ± 15.10 years). Blood samples were obtained from patients at the morning before

surgical intervention. The reference cohort in our case-control study comprised 131 unrelated volunteers (51 males and 80 females with mean age 41.45 ± 9.75 years). All control subjects were without any personal or family history of gliomas and they were randomly recruited from a larger population sample. The study was approved by the Ethics Committee of the University Hospital in Bratislava and a written informed consent was obtained from all examined patients.

The levels of sHLA-G antigens (shed HLA-G1 and secreted HLA-G5) were determined by sHLA-G ELISA assay kit (Exbio, Prague, Czech Republic) according to the manufacturer's instructions.Genomic DNA was isolated from EDTA-treated peripheral blood samples (2 mL) by a modified salting-out procedure (8). HLA-G 5′URR region from – 828 bp to – 5 bp was amplified by forward primer 5′- CACGGAAACTTAGGGCTACGG-3 and reverse primer 5′-GCGTCTGGGGAGAATGAGTCC-3′ as described by Catamo et al. (9). The PCR products were run in 1.0% agarose gel for 20 min and then visualized under UV light. Fragment size of 823 bp was confirmed using the 100 bp DNA ladder (Solis BioDyne, EU). For direct sequence analysis, the PCR products were purified using an EXO SAP-IT kit according to the manufacturer's recommendations (USB, USA) and then sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA). The sequence data were analyzed by SeqScape software (Applied Biosystems) using the reference sequence reported on website http:// www.ebi.ac.uk/imgt/hla/align.html. In 59 patients with gliomas and 131 controls, 16 polymorphisms at the HLA-G 5'URR region have been analyzed: −762C/T (rs1632946), −725C/G/T (rs1233334), −716T/G (rs2249863), −689A/G (rs2735022), −666G/T (rs35674592), −646A>G (rs17875391), −633G/A (rs1632944), −540insG (rs17875392), −533delA (rs370236002), −509C/G (rs17875393), −486A/C (rs1736933), −477G/C (rs1736932), −400G/A (rs17875395), −391G/A (rs17875396), −369A/C (rs1632943), −201G/A (rs1233333). Distribution of HLA-G 14 bp insertion/deletion polymorphism in the HLA-G 3′UTR region was also described. Genotype frequencies fit the Hardy– Weinberg equilibrium in patients with gliomas ($\chi 2 = 0.004 – 0.30$, $P = 0.60 – 0.95$) as well as in controls ($\chi 2 = 0.02 – 3.36$, $P = 0.07 – 0.89$).

Results

1. Basic characteristics of patients and controls are summarized in table 1.

TABLE 1 Characteristics of study group

Parameter	**Gliomas**	**Controls**
	N = 59	N=131
Gender ratio male/female	34/25	51/80
Age (mean ± SD; years)	54.70±15.10	41.45±9.75
Age at onset (mean ± SD; years)	53.36±15.17	-
Grade		
Grade II	18	-

Grade III	12	-
Grade IV	29	-
Primary diagnosis	49	-
Recidivas	10	-
Overall survival (mean±SD, months)	17.44 ± 12.98	-
Grade II	28.03 ± 12.22	-
Grade III	19.09 ± 11.88	-
Grade IV	9.98 ± 8.35	-
Progression free survival (mean±SD, months)	12.72 ± 10.12	-
Grade II	18.00 ± 10.11	-
Grade III	16.95 ± 11.37	-
Grade IV	8.00 ± 7.34	-
IDH1/2 mutated		
Grade II	15	-
Grade III	7	-
Grade IV	0	-

Legend: SD – standard deviation, IDH1/2 - isocitrate dehydrogenase

2. All patients with gliomas had significantly higher values of sHLA-G than healthy controls (P = 0.048, Table 2).

TABLE 2. sHLA-G in 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

3. Among different grades of gliomas, we did not observe differences in plasma sHLA-G.

4. sHLA-G correlated negatively with overall survival in whole group of patients (Spearman r = -0.26, P = 0.04). In glioblastoma subgroup, 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 (median 21.99 vs 46.74 U/ml, P = 0.047).

5. In G. IV we observed significant difference in overall survival when we compared patients with sHLA-G above and below cut off 40 U/ml (P = 0.038, Figure 1). In G. II the difference did not reach significance (P = 0.06).

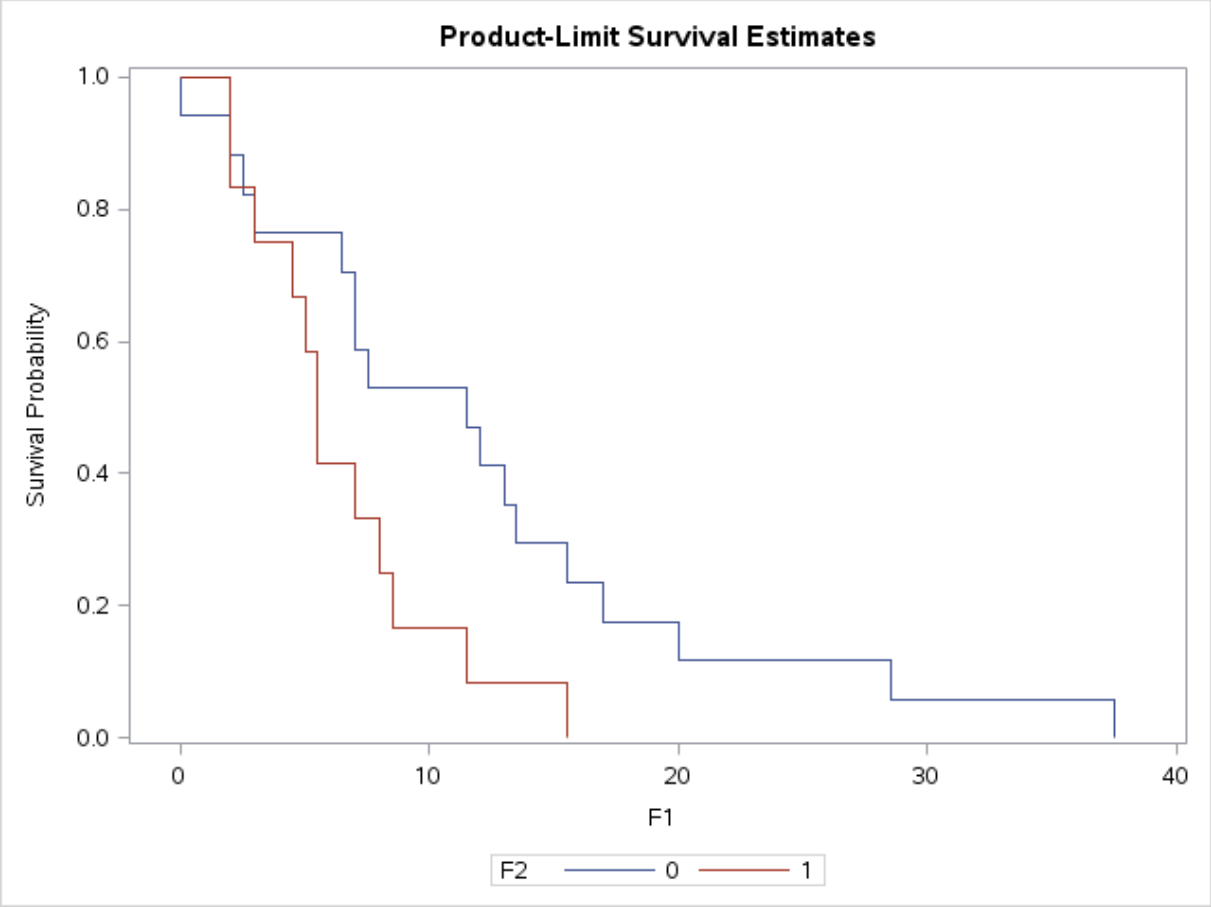

FIGURE 1. Kaplan-Meier survival curves of grade IV according to sHLA-G, 0 – patients with sHLA-G < 40 (N = 17), 1 - patients with sHLA-G > 40 (N = 12) (Test: Log rank P = 0.038)

6. Comparision of HLA-G 5'URR polymorphisms in patients with gliomas and control group

In the HLA-G 5'URR region, 5 SNPs, namely -762C/T, -716T/G, -689A/G, -666G/T, -633G/A, were in strong LD and constituted a haploblock (D'>0.99). The haploblock

consisting of -762T, -716G, -689G, -666T and -633A allele was significantly more frequent in patients with gliomas than in the controls (P = 0.027, OR = 1.68, 95% CI = 1.08-2.61). Also the minor alleles of the -486A/C and -201G/A were significantly more frequent in patients with gliomas than in the controls (-486C: P = 0.033, OR = 1.66, 95% CI = 1.07-2.57, -201A: P = 0.019, OR = 1.73, 95% CI = 1.12- 2.69). Furthermore, -369AC carriers (P = 0.044, OR = 0.96, 95% CI = 0.48-1.93) and -201GA carriers in co-dominant model (P = 0.042, OR = 0.76, 95% CI = 0.38-1.55) had significantly higher frequencies as compared to controls. After adjustment for sex, age and +14 bp ins no statistically significant differences in allele and genotypes frequencies between patients with gliomas and the controls were observed (P = 0.05).
The distribution of the HLA-G 5′URR haplotypes in patients with gliomas and control group was also performed. Phase software estimated 6 HLA-G 5′URR haplotypes in patients with gliomas and 13 HLA-G 5′URR haplotypes in control group. The HLA-G 5′URR-1, URR-3 and 4 were the most frequent haplotypes in patients with gliomas, and HLA-G 5′URR-1, 5′URR-4 and 5′URR-5 were the most frequent haplotypes in control group. The 5'URR-1 haplotype was significantly more frequent in patients with gliomas than in the controls (50% vs 38.17%, P = 0.0403).

7. Association of HLA-G 5′URR polymorphisms with clinical variables in patients with gliomas

The analysis of association between HLA-G 5′URR polymorphisms and clinical variables as age at onset, overall survival and progression free survival (PFS) were performed in subgroups related to glioma grade. In patients with grade IV gliomas we observed that haploblock of -762CT, -716TG, -689AG, - 666GT, -633GA, -486AC, -477GC, -201GA following by -369AC carriers tend to have lower age at onset as compared to other genotype carriers (60.56 ± 8.74 vs 70.00 ± 7.45, P = 0.04). After adjustment for sex and +14 bp ins positivity, statistically significant correlation between above mentioned carriers and lower age at onset mean was also found (P = 0.004). Further findings revealed that after correction for sex and +14 bp ins positivity, statistically significant association of -762CC carriers (constituting haploblock of -716TT, -689AA, - 666GG, -633GG, -486AA, -477GG, -201GG) and -369CC carriers with lower OS and PFS mean as compared to other genotype carriers was determined (P = 0.019 – 0.04).

Discussion

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 (10, 11, 12, 13). Also, some studies found association of higher sHLA-G with worse prognosis (14). 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. Patients with sHLA-G more than 40 U/mL survived significantly shorter than patients with sHLA-G less than 40 U/ml. We suppose that more immuno-suppressive sHLA-G in the peripheral blood inhibits anti-tumor immunity, helps tumor growth, although it promotes faster progression and shorter overall survival. This hypothesis is supported with our finding, that patients with GBM, who survived less than one year had significantly higher values of sHLA-G. In our study the association between HLA-G 5′URR variants and main clinical variables such as age at onset, OS and PFS in patients with gliomas was also evaluated. In

patients with grade IV gliomas we observed that haploblock of -762CT, -716TG, -689AG, -666GT, -633GA, -486AC, -477GC, -201GA following by -369AC carriers tend to have lower age at onset as compared to other genotype carriers (P = 0.04). The impact of HLA-G 5′URR variants on clinical features as OS and PFS in patients with grade IV gliomas was only observed after adjustment for sex and 14bp positivity. To our knowledge, the correlation between HLA-G 5′URR variants and main clinical variables for glioma cases has not been reported until now.
Increased expression of HLA-G in patients with gliomas should be taken into consideration and change therapeutic strategies to prevent its further production. Concerning our results, it seems that some polymorphisms at the 5′URR might have impact on susceptibility to develop gliomas.

Conclusion

This is the first study performing comprehensive association analysis between HLA-G 5' URR polymorphisms and risk of glioma development. We found association of HLA-G 5′URR haploblock consisting of -762T, -716G, -689G, -666T and -633A alleles with susceptibility to develop gliomas. In grade IV glioma patients we observed that haploblock of -762CT, -716TG, -689AG, - 666GT, -633GA, -486AC, -477GC, -201GA following by -369AC carriers tend to have lower age of disease onset as compared to other genotype carriers.

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