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POSSIBLE INTERFERENCE OF HUMAN BETA-HERPESVIRUSES-6 AND -7 IN GASTROINTESTINAL CANCER DEVELOPMENT

A. Sultanova[1,], M. Chistjakovs[1], S. Chapenko[1], S. Donina[1,2], M. Murovska[1]*

[1]Riga Stradins University, August Kirchenstein Institute of Microbiology and Virology, Riga LV-1067, Latvia
[2]Riga Eastern Clinical University Hospital, Latvian Oncology Centre, Riga, LV-1079, Latvia

Aim: **The high incidence of gastrointestinal cancer combined with high mortality from the disease if diagnosed at a late stage, signifies the need for better diagnostic, prognostic and predictive tools. Human beta-herpesviruses have been suggested as possible cofactors in the development of gastrointestinal cancer.** *Methods*: **Sixty five patients with gastrointestinal cancer before surgery and without any treatment were enrolled in this study and divided into two groups depending on lymphocytes' count: I group (n = 35) — lymphocytes > 1400×10^6/L and II group (n = 30) — lymphocytes < 1400×10^6/L. Nested polymerase chain reaction was used to detect latent and active stage of persistent human herpesvirus-6 and -7 infection, laser flow cytometry with monoclonal antibodies — to determine immunological parameters.** *Results:* **Activation of herpesvirus-6 and -7 was more frequently observed in the patients' group with lymphopenia (HHV-6 1/1 (100%), HHV-7 4/8 (50%) and HHV-6 + HHV-7 6/9 (66%); $p < 0.05$). Cellular immune parameters were analysed in immunocompromised II group's patients dependently on beta-herpevirus infection. Although number of leukocytes was higher in patients with active HHV-6/-7 infection ($p = 0.01$), number of lymphocytes CD3^{+}, CD4^{+}, CD8^{+} and CD38^{+} in patients with active HHV-6/-7 infection tended to decrease ($p < 0.0001$, $P = 0.0002$, $p = 0.0001$ and $p < 0.0001$, respectively). However, number of CD19^{+} had tendency to increase ($p = 0.03$).** *Conclusion*: **Activation of herpesvirus-6 and -7 may lead to decrease of lymphocytes total count and develop immunosuppression in patients with gastrointestinal cancer.**
Key Words: **beta-herpesvirus-6, beta-herpesvirus-7, gastrointestinal cancer.**

Gastrointestinal cancer (GIC) usually develops from a benign polyp through an adenoma with dysplasia into a carcinoma with metastatic potential. The late diagnosis of this disease often leads to the high incidence of patients' death. This signifies the need for better diagnostic, prognostic and predictive tools. The emergence of knowledge on the molecular level has gained insight in causes for initiation and progression of tumour development. This knowledge has also revealed the complexity and heterogeneity of the disease, explaining why only few biomarkers are in routine clinical use. GIC is the second and third most commonly diagnosed cancer in the world for women and men, respectively. Approximately half of GIC patients develop metastatic disease [1].

Human herpesvirus-6 (HHV-6) and human herpesvirus-7 (HHV-7) are ubiquitous beta-herpesviruses [2]. Both are widely distributed in the general population and primary infection usually occurs in the early years of life and remains latent in the host for the lifelong period [3]. These viruses can be reactivated in immunosuppressed conditions and can lead to severe complications in patients with solid organ transplantation [4]. Malignancy also is associated with immunosuppression in hematological tumors and in solid organ cancers as well.

HHV-6 and HHV-7 share a high degree of genomic homology and have some similar biological proper-

ties. Thus, these herpesviruses might share a similar oncogenic potential [5]. For both, HHV-6 and HHV-7, main target cells appeared to be CD4^{+} lymphocytes, but natural killer cells, CD8^{+} T cells, macrophages, epithelial, endothelial, neural cells and fibroblasts may also be infected [6, 7].

HHV-6 has immunomodulating properties and is a powerful inducer of cytokines. One important mechanism of HHV-6 pathogenesis is the engagement of the primary viral receptor, CD46, a complement-regulatory cell surface molecule that provides a key link between innate and adaptive immune responses [8]. Recently it was shown that exposure to HHV-6 results in a dramatic inhibition of IL-12 p70 production by differentiated human macrophages in the absence of a productive viral infection, a phenomenon that is likely mediated by CD46 engagement [9]. Other mechanisms of immune deregulation by HHV-6 include defective antigen presentation by dendritic cells and aberrant cytokine production by peripheral blood mononuclear cells, such as an increased secretion of IL-1β, tumor necrosis factor alpha (TNF-α), and IL-10 and a decreased secretion of IL-2 associated with diminished cellular proliferation [10–12]. Persistent, IL-2 regulated, HHV-6 infection of adult T-cell leukemia cells causes T cell leukemia to progress more rapidly, but *in vivo* studies a pathogenic role for HHV-6 in this disease has not been yet confirmed [13]. Virus-induced changes in cytokines secretion can lead to changes in tumor microenvironment and deviation of anti-tumour immune response. HHV-6 may also contribute to cancer circuitously through immune suppression. HHV-6 can directly infect CD4^{+} T-cells and induce apoptosis, as an effective CD4^{+} T cells response is believed to prevent tolerance induction by tumor antigen [14–16].

Despite of HHV-7 and HHV-6 similarities, important differences between these viruses exist, including the fact that HHV-7 binds to the cellular CD4$^+$ molecule and uses this protein as a necessary component of its receptor, while HHV-6 binds to a different receptor. Furthermore, the pathogenesis and sequelae of HHV-7 infection remains very poorly understood [17].

MATERIALS AND METHODS

Patients (n = 65) with histologically confirmed various stages of gastrointestinal cancer (GIC) were aged from 39 to 85 years. The cohort was established with the approval of the Ethics Committee of the Riga Stradins University and all participants gave their informed consent prior to the examination. Blood was drawn before surgery and any antitumor treatment, patients were divided into two groups depending on lymphocyte count: I group (n = 35) — lymphocytes > 1400 x10^6/L and II group (n = 30) — lymphocytes < 1400 x10^6/L. Immunological parameters were determined by Becton Dickinson (USA) laser flow cytofluorimeter using corresponding monoclonal antibodies to lymphocyte subpopulations: CD3$^+$, CD4$^+$, CD8$^+$, CD38$^+$, CD16$^+$, CD19$^+$, CD95$^+$ and CD25$^+$.

Nested polymerase chain reaction (nPCR) was used for the detection of persistent infection (viral genomic sequences in whole blood DNA) and active phase (viral genomic sequences in cell free plasma DNA) of persistent HHV-6 and HHV-7 infection. Total DNA was isolated from 0.5 ml of fresh whole blood by phenol-chloroform extraction. For DNA purification from 200 µl of cell free blood plasma QIAamp Blood Kit (QIAGEN, Germany) was used. The plasma samples were treated with Deoxyribonuclease I before DNA purification. To assure the quality of the whole blood DNA as well as to exclude contamination of plasma DNA by cellular DNA, globin PCR was performed. PCR amplification for the viruses was carried out in the presence of 1 µg of whole blood DNA and 10 µl of plasma DNA (corresponding to 100 µl of plasma). The detection of HHV-6 and HHV-7 DNA was performed according to Secchiero et al. (1995) and Berneman et al. (1992), respectively. Positive (viruses genomic DNA, ABI, USA) and negative (DNA without virus-specific sequences) as well as water controls were included in each experiment.

HHV-6 variants were identified using restriction endonuclease analysis. The restriction enzyme *Hin*dIII (Fermentas, Lithuania), which cuts the 163-bp HHV-6B amplimer into two fragments of 66 and 97 bp, but does not cut the HHV-6A amplimer was used for this purpose.

HHV-6 specific antibody testing in the plasma samples was carried out using HHV-6 IgG ELISA kit (Panbio, Australia) according to the manufacturer's recommendations.

Human TNF-α, IL-1β, sIL 2R: Solid-phase, two-site chemiluminiscent immunometric assay (Immulite, SIEMENS, USA). IL-6: solid-phase, enzyme-labeled chemiluminiscent sequential immunometric assay (Immulite SIEMENS, USA) according to the manufacturer's recommendations. All samples were tested in duplicates.

Statistical difference in the prevalence of latent and active HHV-6 and HHV-7 infection was assessed by Fisher's exact test. Student's *t*-test was used to compare significance in changes of plasma cytokines' levels and cell counts. For the prediction of lymphopenia regression analysis was used.

RESULTS

Comparative analysis of cellular immune parameters in both immunocompetent (lymphocytes > 1400x10^6/L) and immunocompromised (lymphocytes < 1400x10^6/L) GIC patients groups showed significant differences in almost all rates independently of beta-herpesviruses infection. The mean absolute number of lymphocytes in the I group was two times (50%) higher than in the II group (2270 ± 700x10^6/L; 1140 ± 210 x 10^6/L, respectively; *p* = 0.0001) (Table 1). However, the mean number of leukocytes in the II group (7910 ± 1960 10^6/L) was significantly (*p* = 0.002) higher (27%) than in the I group (5830 ± 2210x10^6/L). Comparative analyses of the lymphocyte subsets between the I and II group (CD3$^+$, CD4$^+$, CD8$^+$, CD38$^+$, CD16$^+$, CD19$^+$, CD25$^+$ and CD95$^+$) showed significant decrease of immunological parameters in the II group — approximately two times decrease in comparison with the I group (Table 1).

Table 1. Immunological parameters in the I and II patients groups

Parametrs	Group I Ly > 1400 (n=35)		Group II Ly < 1400 (n=30)		p ≤ 0.05
	Absolute count ± SD	Count, %	Absolute count ± SD	Count, %	
Leu	7910 ± 1960		5830 ± 2210		0.002
Mo	650 ± 170	8.44	440 ± 220	7.87	0.0005
Ly	2270 ± 700	28.83	1140 ± 210	19.88	0.0001
CD3$^+$	1600 ± 580	70.77	780 ± 180	70.77	0.0001
CD4$^+$	880 ± 330	39.29	450 ± 130	39.0	0.0001
CD8$^+$	680 ± 370	29.11	330 ± 120	31.08	0.0001
CD38$^+$	660 ± 300	29.03	320 ± 90	28.4	0.0001
CD16$^+$	460 ± 310	19.69	220 ± 130	19.4	0.0015
CD19$^+$	190 ± 110	8.03	110 ± 60	7.0	0.0018
CD95$^+$	1130 ± 370	50.89	520 ± 130	48.8	0.0001
CD25$^+$	180 ± 150	8.63	80 ± 50	7.5	0.047
CD4$^+$/CD8$^+$	1.58 ± 0.85		1.18 ± 0.57		0.03

Virological studies showed that 44 out of 65 (68%) GIC patients had persistent beta-herpesviruses infection. HHV-6 genomic sequence was detected in 15/65 (23%), when presence of anti-HHV-6 specific IgG class antibodies was detected in 33/65 (51%) patients. HHV-7 genomic sequence was found in 41/65 (63%) patients DNA. Frequency of single HHV-7 persistent infection was significantly higher (*p* = 0.013) in the I group 20/35 (57%) than in the II group 8/30 (27%), when double (HHV-6 + HHV-7) persistent infection was significantly (*p* = 0.046) higher in the II group 9/30 (30%) than in the I group 4/35 (11%). However, single HHV-6 persistent infection was detected only in one I group's patient and in two patients of the II group (Table 2). Nonetheless, HHV-7 genomic sequence was found only in 3 out of 20 (15%) plasma DNA samples of CIG patients of the I group. In contrast, activation of HHV-7 was found in 4 out of 8 (50%) patients of the II group. Furthermore, simultaneous activation of both viruses (HHV-6 + HHV-7) was significantly more often detected in the

II group's patients (6/9 (66%); $p < 0.05$) (Table 2). In all HHV-6 positive DNA samples isolated from GIC patients' white blood cells and plasma HHV-6B variant was identified.

Table 2. Distribution of latent and active beta-herpesviruses (HHV-6 and HHV-7) infection

	HHV-6 latent/active infection	HHV-7 latent/active infection	HHV-6 + HHV-7 latent/active infection	Without HHV-6 or HHV-7 infection
I patients group (n = 35)	1/0	17/3	4/0	10
II patients group (n = 30)	1/1	4/4	3/6	11

Comparative analysis of cellular immune parameters in the I and II group was performed dependently of beta-herpeviruses infection. Each group was subdivided into three subgroups: GIC patients without, with latent and with active HHV-6 and/or HHV-7 infection. In the I group patients with active viral infection had tendency to increase all cellular immunological parameters (leukocytes, monocytes, lymphocytes and $CD3^+$, $CD4^+$, $CD8^+$, $CD38^+$, $CD16^+$, $CD19^+$, $CD25^+$, $CD95^+$) comparing with the subgroups with latent and without HHV-6 and HHV-7 infection. Although number of leukocytes in the II group with active HHV-6/-7 infection was higher than in other subgroups, number of lymphocytes, $CD3^+$, $CD4^+$, $CD8^+$ and $CD38^+$ cells had tendency to decrease. However, number of $CD19^+$ cells had tendency to increase (Table 3). The logistic regression analysis showed that patients with active viral infection have higher risk of lymphopenia (OR 3.33, 95% CI 0.72–15.51; $p = 0.0035$) than patients with latent viral infection (OR 0.33, 95% CI 0.10–1.07; $p = 0.0035$).

There were no significant changes in the serum levels of IL-6, IL-1β, sIL-2R and TNF-α between GIC patients of the I and II group. However, in the I and II group of patients with active viral infection the levels of IL-6 and sIL-2R had tendency to increase, nonetheless, level of TNF-α in the II group was lower than in patients with latent and without viral infection, and also lower in comparison to the I group's patients with active viral infection (Table 4).

DISCUSSION

At the moment there is little information about influence of beta-herpesviruses infection on the course of disease in patients with GIC. Gastrointestinal malignancies are associated with a compromised immune system and viruses, such as immunotropic and immunomodulating HHV-6 and HHV-7 may be able to utilize cellular mechanisms responsible for the immune response inhibition. Modulation of functional properties of host immune factors is an important mechanism of evading the immune response or creating an environment in which the virus can survive. Our results have shown that in GIC patients group with lymphopenia the activation of HHV-6 and HHV-7 infection is significantly more frequent ($p = 0.003$). In all HHV-6 positive patients HHV-6B variant was detected. This corresponds with the data demonstrated by Lempinen et al. [18]. Proportionally balance of lymphocytes subpopulations in both groups was identical, however, lymphopenia was observed in the II group's patients, which could be closely related with the higher rate of activation of beta-herpesvirus infection. Such interaction could lead to the worst outcomes. However, absolute count of leukocytes in the II group patients with active viral infection increases, preferable beta-herpesvirus cell target populations are decreased (lymphocytes absolute count and $CD3^+$, $CD8^+$ and $CD38^+$ subpopulations). Such difference in cell populations could be another evidence of HHV-6 and HHV-7 involvement in this disease progression. Another interesting observation is noticed in natural killers' population ($CD16^+$), which do not increase even in patients with active viral infection. This abnormal anti-viral response could lead to worse progression of co-infections and worst outcomes in GIC patients.

There are no significant changes observed in serum levels of IL-6, IL-1β, sIL-2R and TNF-α between GIC patients of the I and II groups with latent and without HHV-6/-7 infection, but in the II group's patients with active viral infection level of TNF-α is decreased comparing to the other groups. However, it was hard

Table 3. Average count of immunocompetent cells in the I group and II group patients

Parametrs	Without HHV-6/-7			HHV-6/-7 latent			HHV-6/-7 active		
	Group I (n = 10)	Group II (n = 11)	p	Group I (n = 22)	Group II (n = 8)	p	Group I (n = 3)	Group II (n = 11)	p
Leu	7670 ± 1610	5420 ± 2480	0.02	7720 ± 2050	5800 ± 2090	0.03	10030 ± 1420	6310 ± 2110	0.01
Mo	650 ± 130	490 ± 290	0.0007	610 ± 140	320 ± 50	< 0.0001	930 ± 280	420 ± 190	0.003
Ly	2260 ± 870	1130 ± 230	0.0005	2110 ± 490	1230 ± 60	< 0.0001	3400 ± 420	1070 ± 250	< 0.0001
$CD3^+$	1580 ± 790	770 ± 140	0.003	1490 ± 360	870 ± 110	0.0001	2500 ± 270	730 ± 240	< 0.0001
$CD4^+$	890 ± 450	420 ± 120	0.003	850 ± 280	500 ± 50	0.002	1110 ± 240	440 ± 180	0.0002
$CD8^+$	670 ± 520	340 ± 90	0.052	600 ± 180	370 ± 120	0.002	1190 ± 470	290 ± 130	0.0001
$CD38^+$	680 ± 320	330 ± 140	0.004	630 ± 300	310 ± 40	0.006	780 ± 160	300 ± 50	< 0.0001
$CD16^+$	490 ± 330	250 ± 180	0.04	410 ± 320	210 ± 70	0.1	650 ± 190	210 ± 90	0.0001
$CD19^+$	180 ± 90	80 ± 50	0.004	170 ± 70	110 ± 50	0.04	350 ± 280	130 ± 80	0.03
$CD95^+$	1170 ± 460	500 ± 100	0.0001	1040 ± 230	570 ± 160	0.0002	1620 ± 230	510 ± 150	< 0.0001
$CD25^+$	170 ± 100	110 ± 60	0.11	180 ± 180	50 ± 30	0.05	220 ± 90	80 ± 40	0.001
$CD4^+/CD8^+$	1.68 ± 0.85	1.24 ± 0.49	0.16	1.55 ± 0.9	1.31 ± 0.49	0.5	1.37 ± 0.56	1.29 ± 0.65	0.8

Table 4. Levels of IL-1β, IL-6, sIL2R and TNF-α in the I and II group patients*

Patients groups	IL-1β (pg/ml); N < 5.0	IL-6 (U/ml); N = 3.4	sIL2R (U/ml); N = 223–710	TNF-α (pg/ml); N = 8.1
Group I (n = 10) Without HHV-6/-7	< 5.0	4.08 ± 1.23	527 ± 159.58	15.13 ± 5.15
Group II (n = 11) Without HHV-6/-7	< 5.0	5.15 ± 2.76	559.80 ± 379.10	15.58 ± 5.66
Group I (n = 22) HHV-6/-7 latent	< 5.0	5.44 ± 2.99	604.78 ± 242.33	11.62 ± 1.08
Group II (n = 8) HHV-6/-7 latent	< 5.0	5.55 ± 1.91	617.00 ± 173.20	13.83 ± 3.06
Group I (n = 3) HHV-6/-7 active infection	< 5.0	7.20 ± 11.88	851 ± 528.55	14.40 ± 5.31
Group II (n = 11) HHV-6/-7 active infection	< 5.0	6.18 ± 2.91	733.67 ± 388.76	9.78 ± 4.23

*p > 0.05 for all groups.

to compare GIC patients with active viral infection because of higher HHV-6 and HHV-7 distribution in the II group and low presence of active infection in the I group. Despite of this observation, it is possible to suppose important role of beta-herpesviruses in GIC development and decreased level of TNF-α and increased levels of IL-6 and sIL2R in patients with active and persistent virus infection could confirm HHV-6 and HHV-7 immunomodulating properties. Both facts, decrease in lymphocytes' subpopulations and changes in levels of interleukins, could show possible mechanisms of how HHV-6 and HHV-7 influence the course of the disease. First mechanism is direct influence on lymphocytes by infecting them and inducing cell lyses, and second — influence through changes in interleukins expression. Combining studies of both virus and cancer mediated immune suppressive mechanisms will help us to understand the complicated host-tumour interactions. The further investigation is important to evaluate not only changes in pro-inflammatory cytokines but also in anti-inflammatory cytokines expression in patients with GIC. It could explain ways of virus influence on the course of disease and would help to choose the most efficient treatment tactic.

In conclusion, activation of HHV-6 and HHV-7 may lead to decrease of lymphocytes' total count and worsening of immunsuppresion in patients with GIC. High frequency of beta-herpesviruses infection in patients with GIC is contributing into increase of IL-6, sIL-2R and decrease of TNF-α expression levels what could lead to the worse clinical outcomes. Estimation of the viruses-associated impairment of immunological functions may be useful for clinical application to monitoring of GIC patients.

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CONFLICT OF INTEREST

None of the authors of the above manuscript has declared any conflict of interest.

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