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ORIGINAL ARTICLE

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The possible use of the blood serum concentration measurements of sHLA-G in women with endometrial and cervical cancers during radiotherapy as an indicator of the status of the tumour microenvironment

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ABSTRACT

Background: The selective suppression of cytotoxic immune cells constitutes a crucial event in the development of malignancy. This phenomenon increases in accordance with the growth of a tumor and is just one result of the increased expression in the cancer milieu of those proteins, such as human leukocyte antigen G (HLA-G) and its soluble form (sHLA-G). Given that radiotherapy may influence immune system activity, we aimed to measure (sHLA-G) serum levels both before and after the radiotherapy due to endometrial or cervical cancer.

Methods: We assessed the sHLA-G blood serum concentration levels in a group of 43 patients (28 and 15 diagnosed with cervical cancer and endometrial cacer respectively), who received primary or adjuvant radiotherapy. We assessed the blood serum concentrations of the sHLA-G through a series of measurements taken before and four days after the latest radiation dosage using an ELISA kit.

Results: Median serum sHLA-G levels significantly decreased after radiotherapy (5.63 U/ml; range 0.00 – 344.55; vs 5.57 U/ml; 0.00 –94.02; P = 0.045). The changes of sHLA-G levels didn't influence patients' survival. Pretreatment and post-treatment sHLA-G levels were negatively correlated with patients' age (R Spearman = -0.45, P = 0.041; R Spearman = -0.46. P = 0.038).

Conclusions: The detected levels of sHLA-G blood serum concentrations may supply clinically applicable information regarding the status of the tumor microenvironment — that is, the size and the degree of suppression of the tumor environment — where the tumor-immune cell interaction is realized. Finally, this information may also prove helpful in the treatment of cancer.

Key words: Ovarian cancer, endometrial cancer, sHLA-G

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Introduction

The suppression of the immune system constitutes a crucial event in the development of malignancy. It is known that this phenomenon increases in accordance with the growth of a tumour as a result of the increased expression in the cancer milieu of the proteins responsible for the evasion of cancer cells from immune system surveillance. The suppressive environment is related not only to the membrane form of expression, but also to the secretion of the soluble form of these proteins, such as Fas-L [1], RCAS1 [2–3], and HLA-G [4], to the

extracellular matrix of the cancer microenvironment. This profile of the microenvironment is also determined by the infiltration of immune regulatory cells (e.g., Treg) into the cancer microenvironment itself [5] and to the polarization of the tumour microenvironment by an increase in Th2 cytokine (e.g., IL-10) concentration [6]. Tylor *et al.* have demonstrated that the recruitment of Tregs to the cancer microenvironment inhibits an effective antitumor immune response [7]. It has been shown that radiotherapy affects the function of the immune system. Muroyama *et al.* have shown that RT-induced proliferation of Treg cells and the post-RT intratumoral Treg cells have a suppressive function [8]. Generally, the clinical studies suggest that radiotherapy increase the production of Tregs and their recruitment to local tumour microenvironment [9].

The presence of these factors in the tumour microenvironment may be discerned in the cancer milieu. Furthermore, the levels of its expression or the concentrations of its soluble forms can be determined not only in the tumour microenvironment but also in the peripheral blood. Since these factors are crucial for the development of the phenomenon, the possibility of assessing the levels in women treated for gynaecological malignancies would seem to constitute a clinically applicable indicator of the status of the tumour microenvironment — that is, the size and the degree of suppression of the tumour environment. Most likely, the use of the information deriving from the interactions between cancer and its stroma and the immune cells in the tumour microenvironment will spur improvement in the therapy for gynaecological malignancies. As of now, treatment for endometrial cancers remains a clinical problem [10].

We focused on our studies on the cancer microenvironment and on the proteins present there and deriving from the cancer milieu. Most likely such proteins like HLA-G can be used as biomarkers not specifically linked with the particular type of gynaecological malignancy, as they have also been found under normal physiological conditions in women's reproductive tracts (e.g., in the feto-maternal interface) [11]. The blood serum profiles of these biomarkers may supply interesting information for clinicians, such as the fact that developing cancer modifies its own microenvironment.

HLA-G is an antigen whose participation in the regulation of the immune system has been well documented [11]. HLA-G is one of the proteins involved in the regulation of the interaction of the tumour and immune cells that takes place in the microenvironment of a growing tumour [12, 13]. HLA-G is believed to protect the target cells that are deficient in HLA class I antigens from NK-dependent lysis by interacting on their surfaces with killer-inhibitory receptors. Tumour cells may, therefore, be excluded from the host immune response. Expression of the suppressive molecule HLA-G differs from that found in other types of malignancies. In general, an increased expression of HLA-G is known to be associated with disease progression not only in ovarian [14], endometrial [15], and breast [16] cancers, but also in non-gynaecological types of malignancies, such as bladder cancers [17], and retinoblastoma [18]. Park *et al.* have demonstrated that cells can generate the soluble HLA-G (HLA-G1 and HLA-G5) by the dual mechanism of alternative splicing and proteolytic shedding and that the soluble form of HLA-G is able to inhibit the lytic activity of NK cells [11]. The concentration of the soluble form of sHLA-G in the peripheral blood could demonstrate the level of suppression by the tumour environment. Ben Yahia *et al*. have demonstrated the growth of sHLA-G in early stages (Stages I and II) as well as the correlation with grading of endometrial cancer. The alteration of the level of sHLA-G was associated with the rapid spread of the disease [19].

The presence in the peripheral blood proteins such as sHLA-G might be related to the suppressive influence of cancer cells on the immune system. This information detected in peripheral blood is valuable and applicable especially from a clinical point of view. For this reason, we decided in our study to evaluate the sHLA-G blood serum concentration levels both before and after the radiotherapy in patients treated for cervical and endometrial carcinomas.

Material and methods

Human subject

In the case of early-stage cervical cancer patients (up to and including IB1 according to FIGO classification) a radical hysterectomy and adnexectomy with pelvic and paraaortic lymphadenectomy were performed followed by brachytherapy and external beam radiation. More advanced stages were treated with primary radiotherapy with or without concurrent chemotherapy. Patients with endometrial cancer were treated surgically in all cases and simple, extrafascial hysterectomy with bilateral ovariectomy with pelvic lymphadenectomy was performed. All of the analyzed patients with endometrial cancer were treated with adjuvant external beam radiation and brachytherapy with or without chemotherapy. The mean age of the patients included was 58 (range 35–84 years). The patients had undergone treatment in the Gynecologic Oncology Department of the M. Sklodowska-Curie Memorial Institute or in the Gynecology and Oncology Department of the Lukaszczyk Oncological Center, respectively in Krakow and Bydgoszcz between January 2007 and September 2010. The patient's consent was obtained in each case. Prior to the study, the approval of the Jagiellonian Uni-

versity Ethical Committee (KBET/135/B/2007) was also obtained. Information on all the patients who died was retrieved from the database of the Kujawsko-Pomorski and Malopolski regional office of the National Health System of Poland. We have analyzed long-term outcomes after radiotherapy regarding overall survival (OS).

ELISA

The blood was collected to a serum collection tube both directly prior to radiotherapy and on the fourth day following the last radiation dosage. A clot was allowed to form at room temperature for 30–60 minutes. The tube was placed on ice for 30 minutes in order to contract a clot. The serum samples were then centrifuged at 3000xg for 10 minutes at room temperature. The supernatants 1.0–2.0 ml were collected and stored at -80°C. The analysis of sHLA-G concentration in the serum samples was performed in the Department of Analytical Biochemistry, Faculty of Biochemistry, Biophysics, and Biotechnology, Jagiellonian University. The soluble human leukocyte antigen-G (sHLA-G) was detected using the sHLA-G sandwich ELISA kit (BioVendor-Exibo, Czech Republic). Briefly stated, the blood plasma samples were diluted twice and incubated for 1 hour in the 96-well microplate precoated with the monoclonal anti-sHLA-G antibodies. Following incubation, the wells were washed and then filled with the monoclonal anti-human beta-2-microglobulin antibodies labeled with horseradish peroxidase. After an additional 1 hour of incubation, the wells were again washed, and the colour reaction was developed using tetramethyl benzidine (TMB) substrate. The absorbance values were measured at 450 nm on a microplate reader followed by the calculation of the sHLA-G concentrations. The assay was calibrated using a set of sHLA-G standards provided by the producer of the kit.

Statistical analysis

The distribution of variables in the study groups of women checked with the use of the Shapiro-Wilk test showed that each of the women was different from normal. Pre- and postoperative sHLA-G concentrations levels were analyzed using the Wilcoxon test. The statistical significance in the levels of sHLA-G, both pre- and post treatment, between cervical cancer and endometrial cancer patients groups was determined by the Mann-Whitney Test. The Mann-Whitney test was also used for the calculation of differences related to FIGO stage and cancer grade. For survival evaluation, Kaplan-Meier curves analysis was performed.

Results

The statistically significant differences in sHLA-G blood serum concentration level were identified before radiotherapy and on the fourth day following the collection of the last dosage of radiotherapy (Median 5.63 U/ml; range 0.00 – 344.55; vs 5.57 U/ml; 0.00–94.02; p = 0.04, respectively). The results are summarized in Table 2.

No statistically significant differences were identified in the sHLA-G blood serum concentration levels with respect to the clinicopathological parameters, such as FIGO stage and tumour grade. Similarly, there were no differences in pre- and post-treatment sHLA-G levels between cervical and endometrial cancer patients. Pre-treatment sHLA-G levels in the sera of patients with I and II stage disease was not significantly different from patients with stage III and IV disease (5.63 U/ml, range 0–71.82, vs. 5.45 U/ml, range 0–344.55 U/ml, P = 0.735). Similarly, the difference in post-treatment levels of sHLA-G was not statistically significant (5.75 U/ml, 0–41.28 vs 5.09 U/ml, 0–94.02, P = 0.474). The difference in sHLA median levels between well and moderately differentiated tumors was not different from poorly differentiated tumors, both in pre- and post-treatment evaluation (5.63 U/ml, 0–71.82 vs. 5.45 U/ml, 0–344.55; P = 0.71 and 5.75 U/ml, 0–41.28 vs. 5.09 U/ml, 0–94.02 U/ml, P = 0.622).

We have found significant, negative correlation between patients' age both pre- and postreatment and sHLA-G levels (R Spearman = -0.45, P = 0.041; R Spearman = -0.46. P = 0.038 respectively).

No statistically significant differences were identified in the sHLA-G blood serum concentration levels before and after radiotherapy with respect to a long-term outcome. When patients were divided into two groups: patients with decreased or stable post-treatment sHLA-G levels (Group 1, n = 31) and patients with increased post-treatment sHLA-G levels (Group 2, n = 12), there were no statistical significant difference in patients' survival (1592 vs. 657 days, P = 0.60; Figure 1).

Discussion

We have found statistically significant differences between the levels of the blood serum concentrations of sHLA-G as measured before and after radiotherapy in patients treated for cervical and endometrial carcinomas. Radiotherapy induces single- and double-stranded DNA breaks leading to apoptosis [20], but it has been shown that RT affects the immune system activity, including the induction of a systemic antitumor response, with a pro-inflammatory activity and an abscopal effect [21, 22]. RT has also been demonstrated

Table 1. Clinicopathological characteristics and treatment modalities of the patients

In this study, we analyzed the blood serum samples obtained from 43 patients, including No	Age	Cancer	FIGO	Histopathology	Grade	Surgery	Chemo-therapy
1	50	Cervical cancer	IB1	squamous cell carcinoma	3	Yes	No
2	46	Cervical cancer	IB1	squamous cell carcinoma	3	Yes	Yes
3	35	Cervical cancer	IB2	squamous cell carcinoma	2	No	No
4	50	Cervical cancer	IIA	squamous cell carcinoma	2	No	Yes
5	51	Cervical cancer	IIA	squamous cell carcinoma	3	No	Yes
6	48	Cervical cancer	IIB	squamous cell carcinoma	2	No	No
7	65	Cervical cancer	IIB	adenocarcinoma	2	No	Yes
8	65	Cervical cancer	IIB	squamous cell carcinoma	2	No	Yes
9	52	Cervical cancer	IIB	squamous cell carcinoma	2	No	Yes
10	52	Cervical cancer	IIB	adenocarcinoma	1	No	Yes
11	62	Cervical cancer	IIB	squamous cell carcinoma	3	No	Yes
12	42	Cervical cancer	IIB	squamous cell carcinoma	3	No	Yes
13	48	Cervical cancer	IIIB	squamous cell carcinoma	2	No	No
14	63	Cervical cancer	IIIB	squamous cell carcinoma	1	No	No
15	58	Cervical cancer	IIIB	squamous cell carcinoma	3	No	Yes
16	72	Cervical cancer	IIIB	squamous cell carcinoma	3	No	Yes
17	48	Cervical cancer	IIIB	squamous cell carcinoma	2	No	No
18	52	Cervical cancer	IIIB	squamous cell carcinoma	2	No	Yes
19	49	Cervical cancer	IIIB	adenocarcinoma	2	No	Yes
20	47	Cervical cancer	IVB	squamous cell carcinoma	2	No	Yes
21	58	Cervical cancer	IIIB	squamous cell carcinoma	3	No	Yes
22	43	Cervical cancer	IIIB	squamous cell carcinoma	2	No	Yes
23	52	Cervical cancer	IIB	squamous cell carcinoma	2	No	Yes
24	68	Cervical cancer	IIA	squamous cell carcinoma	2	No	Yes
25	62	Cervical cancer	IIB	squamous cell carcinoma	3	No	Yes
26	46	Cervical cancer	IB1	squamous cell carcinoma	3	Yes	Yes
27	68	Cervical cancer	IIA	squamous cell carcinoma	2	No	Yes
28	48	Cervical cancer	IIIB	squamous cell carcinoma	2	No	No
29	66	Endometrial cancer	IB	endometrioid adenocarcinoma	3	Yes	Yes
30	79	Endometrial cancer	IB	endometrioid adenocarcinoma	2	Yes	No
31	74	Endometrial cancer	IB	endometrioid adenocarcinoma	3	Yes	Yes
32	52	Endometrial cancer	IB	endometrioid adenocarcinoma	2	Yes	No
33	61	Endometrial cancer	IIIA	endometrioid adenocarcinoma	2	Yes	Yes
34	66	Endometrial cancer	IIIB	endometrioid adenocarcinoma	1	Yes	No

→

Table 1 cd. Clinicopathological characteristics and treatment modalities of the patients

In this study, we analyzed the blood serum samples obtained from 43 patients, including No	Age	Cancer	FIGO	Histopathology	Grade	Surgery	Chemo-therapy
35	74	Endometrial cancer	IIIC1	endometrioid adenocarcinoma	3	Yes	Yes
36	61	Endometrial cancer	IIIB	endometrioid adenocarcinoma	2	Yes	No
37	52	Endometrial cancer	II	endometrioid adenocarcinoma	1	Yes	No
38	46	Endometrial cancer	IIIA	serous adenocarcinoma	3	Yes	Yes
39	78	Endometrial cancer	IIIA	serous adenocarcinoma	3	Yes	Yes
40	84	Endometrial cancer	II	undifferentiated carcinoma	3	Yes	No
41	70	Endometrial cancer	II	endometrioid adenocarcinoma	1	Yes	No
42	74	Endometrial cancer	IIIC1	endometrioid adenocarcinoma	3	Yes	Yes
43	70	Endometrial cancer	II	endometrioid adenocarcinoma	1	Yes	No

Table 2. The difference in pre- and post-treatment serum sHLA-G levels

	Median	Range	P-value
Pretreatment	5.63 U/ml	0.00–344.55	P = 0.04
Post-treatment	5.57 U/ml	0.00–94.02	

to exert an immunosuppressive effect, increased levels of functionally active Treg lymphocytes were detected following radiotherapy [23]. Ionizing radiation has also been demonstrated to modulate the HLA-G expression. Michelin *et al*. have demonstrated that irradiation downregulated cell surface and total HLA-G levels and increased sHLA-G1 in the medium of the melanoma cell line. Authors concluded that radiotherapy might induce a proteolytic cleavage of this molecule [24]. Most probably decreasing serum level of sHLA-G observed after radiotherapy is linked with reduction of the tumour mass that the patients underwent during surgery before adjuvant radiotherapy.

The suppression of the immune system constitutes a crucial event in the development of malignancy, particularly in cases of cancer relapse. Surgery, chemo- and radiotherapy all have different effects on tumour and immune cell interaction [25, 26]. Since, on the one hand, the immune system can demonstrate anti-tumour activity, but, on the other hand, can promote tumour growth, the degree of the suppressive influence of cancer cells on the immune system may be able to determine the success of the treatment for a cancer relapse [2]. Nevertheless, it is not common practice to evaluate the suppressive influence of cancer cells on the immune system. This detection could be helpful in monitoring treatment processes and might reflect the influence of this treatment on the restoration of proper immune system activity. sHLA-G, however, has been observed in blood sera of patients with gynaecological malignancies [15]. Furthermore, sHLA-G has not yet been studied in relation to the applied surgery in cases of gynaecological malignancies.

HLA-G expression has been observed in endometrial cancer and can be compared with the expression reported in various other malignances. In immunohistochemical staining, HLA-G expression varied between 40% and 55% according to different studies [15, 27]. This discrepancy could be the result of the different characteristics of the patients included in the study, such as being in an advanced stage of the disease or having a tumor with non-endometrioid histology. Barrier *et al.* found a correlation between HLA-G expression and increasing FIGO stage which could serve as a pre-operative indicator of dissemination [15]. Contrary to our study we did not observe a correlation between the FIGO stage and the blood serum concentration levels of sHLA-G. Most likely this has to do with the character-

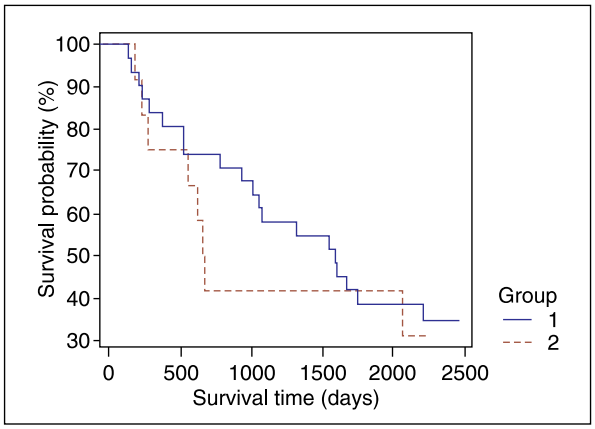

Figure 1. Kaplan-Meier survival curves for analyzed patients. Group 1: patients with decreased or stable post-treatment sHLA-G levels; Group 2: patients with increased post-treatment sHLA-G levels (1592 vs 657 days, P = 0.60)

istics of the patients included in the study, where most patients have advanced diseases [11].

HLA-G expression was associated with disease progression in patients with cervical cancer. In the study of Li *et al.*, the expression of HLA-G molecule gradually increased from preinvasive stages to advanced cancers, indicating the role of HLA-G in tumour progression [28]. Additionally, Dong et al., indicates, that the HLA-G expression is related to HPV16/18 infection [29]. However, tissue expression of HLA-G may not directly reflect serum levels of soluble HLA-G. Similarly to our study, in the paper by Samulels *et al.*, patients survival was not influenced by serum sHLA-G levels. Additionally, the authors also did not find any association between sHLA-G levels and clinicopathological characteristic of cervical cancer [30].

This serial method of measurement may help to reveal the relationship between the applied therapy and the size and degree of the suppression of the tumour environment. Furthermore, such results could indicate that a change in therapy is needed; it could also provide a strong, clinical indication and foundation for the earlier application of molecular therapies, such as immunotherapy.

Conclusions

The detected sHLA-G blood serum concentrations may supply clinically applicable information regarding the status of the tumour microenvironment — that is, the size and the degree of suppression of the tumour environment — where the tumour-immune cell interaction is realized. Finally, this information may also prove helpful in the treatment of cancer.

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Statement of competing interests

The authors declare no competing financial interest and no conflicts of interests.

List of abbreviations

HLA-G — uman leukocyte antigen G
sHLA-G — Soluble Human Leukocyte Antigen-G
RCAS1 — receptor-binding cancer antigen expressed on SiSo cells
Treg — Regulatory T cells
RT — radiotherapy

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