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RESEARCH ARTICLE

Circulating inflammatory markers in cervical cancer patients and healthy controls

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ABSTRACT

There is increasing evidence that host inflammatory responses play an important role in the development and progression of cancers. There are some data that cancer is associated not only with inflammation at the site of the lesion, but also with dysregulations of the host overall systemic immune response. In the case of cervical cancer, inflammation is an important factor associated with the development, progression, and potential metastasis of the disease. What is unclear still in the potential for modifications of host responses to human papillomaviruses (HPV) – a known causative agent of CC, that could be induced by cigarette smoking. In particular, it remains to be determined how the inflammation induced by HPV infection could impact on CC incidence/severity. In this prospective study, serum levels of 10 cytokines were evaluated using Multiplex and ELISA assays. The samples were the sera of 43 CC patients and 60 healthy (NILM) controls. All outcomes were evaluated in relation to host HPV and to their smoking status. The results in indicated that serum sTREM-1, TNFα, IFNβ, IL-1β, and IL-6 levels were significantly increased in CC (HPV+) patients compared to healthy NILM controls. A similar trend was observed for IL-10 and IL-2 levels. Within the two groups, differences in cytokine levels between smokers and never smokers were not remarkable. The findings here support the hypothesized role of systemic inflammation in the pathophysiology of CC.

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Introduction

Cervical cancer (CC) remains a major health problem worldwide, especially in Lithuania: a total of 6399 deaths from CC were reported in Lithuania from 1987 to 2016 (Everatt and Intaitė 2018). The high-risk human papillomaviruses (HR-HPV), known as a major risk factor for CC, may induce pre-cancerous cervical lesions. The HPV itself can also modulate host immune responses and, so in turn, influence lesion progression from carcinoma *in situ* (CIS) to full – on CC (Gunnell et al. 2006). For example, higher levels of interleukin (IL)-10 relative to those of tumor necrosis factor (TNF)-α were observed in HPV-infected women with different types of cervical lesions; these findings appeared to reflect a down-modulation of the host immune response to HPV-related lesions (Ali et al. 2012). Still though, an HR-HPV infection alone is not sufficient to induce CC development. In fact, the vast majority of HR-HPV infected women never develop CC because an adequate immune response can control the infection and prevent any induction/formation of pre-cancerous lesions (Insinga et al. 2011). This can allow one to suggest that additional factors act in conjunction with the actual HPV to influence the host risk of CC development.

It has been found that smoking has an impact on the development of CC. It was reported that smoking helps the HPV to survive and promotes the progression of the viral infection (Roura et al. 2014). There are also data indicating that there is a synergism between duration of cigarette smoking (pack-years) and HPV16 in CIS development (Gunnell et al. 2006). While there are clear associations between invasive CC and current smoking status, intensity, pack-years, and/or time-since-quitting, these associations were found to not be related to the host HPV status (seropositive [HPV^+] vs. seronegative [HPV^-] women) (Roura et al. 2014). Thus, controversy remains with regards to if smoking affects HPV resistance/induced inflammation (in the context of CC formation/severity) OR if HPV impacts on the effects from smoking itself on induction of CC. However, the study undertaken here does not delve into this issue of the impact of smoking itself.

There is increasing evidence that the host inflammatory response plays an important role in the development and progression of cancers. Cancer is associated not only with inflammation at the site of the lesion, but also with the overall systemic immune response (Todoric et al. 2016). Studies have shown that a reduction of inflammation is effective in the treatment and prevention of progression of a variety of cancer types. It has been postulated that smoking affects the inflammation via different stimulatory and suppressor mechanisms (Lee et al. 2012). It is reported that reactive oxidant substances (ROS), well-known cigarette smoke constituents, stimulate inflammatory interleukin (IL)-8 and/or tumor necrosis factor (TNF)-α gene activation, followed by the secretion of these inflammatory mediators which promote chronic immune cell recruitment and inflammation. But the different mechanisms and effects are known also – smoking suppresses T-helper (T_H) Type 1 responses, but

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exaggerate the T_H2 type inflammation via modifications of immune cell polarization (Lee et al. 2012; Todoric et al. 2016). The major mechanism through which smoking promotes cancer is inflammation-related, but the exact etiopathogenetic mechanisms of inflammation in CC are not known yet.

To obtain a better understanding of the role of inflammation in CC, in the study reported here, serum levels of a panel of inflammatory markers were evaluated in CC patients and compared to levels in healthy control counterparts.

Materials and methods

Patients and study design

The study population was composed of women who visited the Department of Obstetrics and Gynecology at the Hospital of Lithuanian University of Health Sciences (Kaunas). Those women with autoimmune diseases, active or chronic infections, cardiovascular diseases, connective tissue diseases, a history of malignant tumors, who were pregnant, and/or were <18 years-of-age were excluded. Any subject who previously received immunosuppressive treatment, radiotherapy, and/or chemotherapy was also excluded. The study was approved by the Kaunas Regional Biomedical Research Ethics Committee (No. BE-2-80/2018). Written consent was received from all subjects upon inclusion in the study.

Of the final 103 study patients acquired, 43 were histologically shown to have CC and 60 were healthy, i.e. cervical cytology tests negative for intra-epithelial lesions/malignancy (NILM) [confirmed by liquid based cytology test (SurePath, Becton Dickinson, Burlington, NC) and reported according to the 2014 Bethesda System formed control group]. Questionnaires were provided to each woman on recruitment. Socio-demographic and lifestyle factors were obtained as was smoking status (self-reported). Nonsmokers were defined as never-smokers, otherwise, subjects were classified as smokers (past and current smokers were included in the same group).

Quantification of serum cytokines

Venous blood samples were drawn from each study patient before any procedures, i.e. the surgery and cancer treatment. Each blood sample was allowed to clot at room temperature and serum was then isolated and stored at −80 °C until used in analyses. The serum concentrations of nine different cytokines, e.g. interferon (IFN)-β, IFNγ, IL-1β, IL-2, IL-6, IL-10, IL-12p70, lipocalin-2 (LCN2), and soluble triggering receptor expressed on myeloid cells (sTREM)-1 were quantified via a Magnetic bead-based multiplex assay (Human Cytokine Premixed Multi-Analyte Kit, R&D, Minneapolis, MN) and a Luminex® 100 Analyzer (Luminex Corp., Austin, TX), according to manufacturer instructions. Each sample was analyzed in triplicate. A commercial ELISA kit (DIAsource, Louvain-la-Neuve, Belgium) was used to measure serum TNFα levels.

HPV detection and genotyping

Cervical samples (biopsy from CC patients, liquid-based cervical samples from healthy controls) were obtained for genomic DNA isolation and HPV status determination. Polymerase Chain Reaction (PCR)-based Multiplex HPV genotyping kits (DiaMex, Heidelberg, Germany) were used (according to manufacturer instructions) to detect/differentiate Human Papillomavirus (HPV) 24 genotypes (Types 6, 11, 16, 18, 26, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, and 82) in each sample.

Statistical analysis

A Kolmogorov–Smirnov test was used to determine distribution of all quantitative data. All data were found to be non-normally distributed and were then compared using a Mann–Whitney *U*-test and a Kruskal–Wallis test. A Chi-square and a Fisher exact test (for small sample size) were used to determine whether a relationship existed between qualitative data. Proportions were compared using a z-test. Significance in all cases was accepted at $p < 0.05$. All analyses were performed using SPSS 23.0 software (IBM, Armonk, NY).

Results

A total of 103 women were classified into two groups, CC patients and an NILM group (healthy controls). Table 1 shows participant socio-demographic and behavioral data. The mean age of the CC patients and NILM groups did not differ significantly ($p = 0.114$). HPV was found in tissue biopsies of all CC patients; analysis of HPV in liquid-based cervical samples from NILM women revealed 15.0% were HPV^+. NILM women more often were city residents and CC patients more frequently were villagers (respectively, $p = 0.030$ and $p = 0.019$). Education status influenced HPV and CC presence rates; a significantly higher percentage of HPV^+ and CC^+ women were found in groups without higher (primary/basic/secondary) education and vice versa, i.e. healthy women more often had university educations. The occurrence of CC and HPV was detected significantly more often in widowed women ($p < 0.001$).

Table 1. HPV, socio-demographic and behavioral characteristics of CC and NILM groups.

	n (%)		
Parameter	CC group ($n = 43$)	NILM group ($n = 60$)	*p* Value
Age (yr)[a]	52.2 ± 12.2	48.9 ± 10.6	0.114
HPV status			
HPV^+	43 (100)	9 (15.0)	<0.001
HPV^-	0 (0)	51 (85.0)	
Smoking status			
Non-smoker	24 (55.8)	51 (85.0)	0.001
Smoker	19 (44.2)	9 (15.0)	
Residency			
City	21 (48.8)	42 (70.0)	0.030
Town	7 (16.3)	9 (15.0)	0.086
Village	15 (34.9)	9 (15.0)	0.019
Education			
Primary	3 (7.1)	0 (0)	*0.067
Basic (lower secondary)	5 (11.9)	1 (1.7)	0.031
Secondary	16 (38.1)	4 (6.7)	<0.001
Higher (non-university)	11 (26.2)	12 (20.0)	0.462
Higher (university)	7 (16.7)	43 (71.7)	<0.001
Marital status			
Married	20 (46.5)	39 (65.0)	0.061
Unregistered marriage	4 (9.3)	5 (8.3)	0.864
Divorced	6 (14.0)	13 (21.7)	0.320
Widow	11 (25.6)	1 (1.7)	<0.001
Single	2 (4.7)	2 (3.3)	0.733

CC: Cervical Cancer group; NILM: healthy women with cytology tests negative for cervical intra-epithelial lesions or malignancy.
[a]Data shown are mean ± SD.
*Fisher exact test was employed for the small sample size.

A total of 28 (27.2%) study women reported they were current or past smokers. Smoking was significantly more prevalent in the CC patients compared to among the NILM ($p < 0.001$; Table 1). The proportion of women who smoked > 20 years did not significantly differ between the CC and NILM women (8/19 [42.1%] vs. 1/9 [11.1%]).

To characterize patient inflammatory status, serum levels of a variety of inflammatory markers were assessed. This study found that among CC patients (vs. NILM controls), there were significantly higher systemic levels of TNFα, IFNβ, IL-1β, TREM-1, and IL-6 (Table 2). A similar trend was observed with regards to levels of IL-10 and IL-2. Serum levels of all the other markers evaluated did not differ between the two groups.

To better understand any relationship between smoking, inflammation, and CC development, serum inflammatory marker levels in patients of varying smoking status were compared. These analyses found only a tendency for increases in serum IL-1β and LCN 2 in CC smokers compared to in CC never-smokers, but these changes were not significant (Table 3). The study did not find significant differences between CC smokers and never-smokers, nor between NILM smokers and never-smokers, in expression of the other evaluated inflammatory markers. There were no differences in expression either when comparing CC smokers vs. NILM smokers or their respective never-smokers.

Discussion

The present study revealed a presence of systemic inflammation in CC patients, i.e. serum TNFα, IFNβ, IL-1β, TREM-1, and IL-6 levels were significantly higher in patients with CC compared to healthy (NILM) controls. A similar trend was seen for IL-10 and IL-2. Differences between IL levels in smokers vs. never smokers were not pronounced. A trend to increased serum IL-1β and LCN 2 levels was noted in CC smokers compared to CC never smokers.

An association between CC and HPV was confirmed in the present study. Specifically, HPV was detected in tissue biopsies of all the CC patients, whereas only a minority (≈15%) of NILM women were found to be HPV-infected. Other authors have reported a high prevalence of HPV in line with our data and have qualified HPV as an important risk factor which is capable to cause precancerous intraepithelial lesions of the cervix, to escape from host immune responses and to influence the progression from CIS to CC (Gunnell et al. 2006; Lukac et al. 2018). Nevertheless, accumulating evidence suggests that HPV is not the only factor responsible for the development of CC. Failure to develop an effective local/systemic immune response can result in a persistent infection and an increased risk of malignant changes among cervical cells (Ali et al. 2012; Lee et al. 2012; Roura et al. 2014; Todoric et al. 2016). While altered immune responses in HPV-infected patients are probably related not only to the presence and actions of HPV, other host factors, such as a smoking, might also play significant roles that ultimately impact on HPV-related induction of CC.

While tobacco use has been associated with different types of cancer (Todoric et al. 2016), in the context of CC, it has been reported the daily cigarette smoking and smoking duration each increase the risk for CC directly. The present study showed that smoking was significantly more prevalent in CC patients compared to NILM women. Such data are consistent with many other studies that reported smoking as a risk factor of importance in women with cervical lesions (Gunnell et al. 2006; Xi et al. 2009; Roura et al. 2014; Lukac et al. 2018). Of note, Roura et al. (2014) reported that all measures related to smoking (e.g. smoking status, duration, intensity, and pack-years, as well as time-since-quit smoking) were associated with CIN3/CIS and invasive cervical cancer. The significance of smoking duration was not detected in the current study. The relative incidence of long-term (>20 yr) smoking did not significantly differ between the CC and healthy smokers. This discrepancy between the other studies cited above and the current only might be a result of a "too-small" subgroup of healthy smokers being included in the current study. While the existence of a link between smoking and inflammation is beyond the question, the mechanisms underlying that relationship remain unclear in many diseases, including CC.

Cytokines have been shown to be associated with most neoplastic tissues and may have play a role in cell transformation,

Table 2. Serum levels of inflammatory markers in CC and NILM women.

	Median value, pg/ml (25th percentile; 75th percentile)		
Marker	CC group ($n = 43$)	NILM group ($n = 60$)	p Value
TNFα	7.50 (5.60; 11.53)	5.40 (4.43; 6.20)	<0.001
IFNβ	102.88 (88.55; 114.83)	93.33 (78.99; 102.88)	0.032
IFNγ	1756.29 (1712.56; 1790.31)	1751.43 (1693.03; 1784.84)	0.164
IL-1β	162.78 (127.79; 170.41)	131.07 (105.93; 166.05)	0.009
IL-10	1110.00 (652.64; 1262.68)	919.53 (568.76; 704.90)	0.078
IL-12p70	686.36 (667.82; 709.53)	695.63 (667.82; 704.90)	0.788
IL-2	576.43 (556.65; 585.55)	561.22 (530.80; 583.65)	0.064
LCN 2	14878.4 (1266.7; 40819.6)	8922.2 (1157.3; 31924.5)	0.112
TREM-1	391.27 (228.10; 472.32)	302.66 (130.84; 352.10)	0.001
IL-6	37.93 (3.61; 116.29)	0.00 (0.00; 2.15)	<0.001

CC: Cervical Cancer group. NILM: healthy women with cytology tests negative for cervical intra-epithelial lesions or malignancy.

Table 3. Inflammatory markers in CC and NILM women groups as function of smoking status.

	CC median, pg/ml (25th; 75th percentile)			NILM Median, pg/ml (25th; 75th percentile)		
Marker	Smoke	Never-smoke	p Value	Smoke	Never-smoke	p Value
TNFα	8.9 (6.4; 12.1)	7.0 (4.4; 9.0)	0.138	5.1 (4.4; 6.1)	5.4 (4.4; 6.2)	0.844
IFNβ	102.9 (95.7; 114.8)	100.5 (86.6; 116.8)	0.659	93.3 (77.8; 100.5)	93.3 (79.0; 102.9)	0.648
IFNγ	1770.9 (1741.7; 1790.3)	1734.4 (1712.1; 1789.7)	0.178	1749.0 (1705.2; 1784.2)	1753.9 (1693.0; 1785.4)	0.804
IL-1β	165.0 (149.6; 175.9)	160.0 (114.9; 167.1)	0.074	154.0 (112.5; 167.1)	130.0 (105.9; 162.8)	0.724
IL-10	1140.7 (861.4; 1338.9)	1033.9 (622.1; 1194.0)	0.186	988.2 (572.6; 1251.2)	862.3 (566.9; 1281.7)	0.736
IL12p70	695.6 (677.1; 734.2)	686.4 (650.8; 702.6)	0.128	691.0 (679.4; 714.2)	695.6 (667.8; 704.9)	0.491
IL-2	576.4 (565.8; 585.6)	575.7 (550.5; 593.2)	0.769	547.5 (524.0; 579.5)	564.3 (532.3; 587.1)	0.258
LCN 2	31728.1 (14140.2; 41640.8)	11643.9 (1097.2; 37777.6)	0.094	13259.1 (2214.1; 33838.5)	7836.7 (1139.3; 32040.2)	0.942
TREM-1	394.5 (305.0; 502.6)	339.4 (147.0; 419.2)	0.126	325.35 (134.52; 354.53)	299.4 (130.8; 351.3)	0.474
IL-6	37.9 (5.6; 98.8)	39.0 (0.00; 127.2)	0.979	0.0 (0.0; 19.4)	2.0 (1.0; 2.0)	0.958

CC: Cervical Cancer group; NILM: healthy women with cytology tests negative for cervical intra-epithelial lesions or malignancy.
CC: Smoker, $n = 19$; never-smoker, $n = 24$. NILM: Smoker, $n = 9$; never-smoker, $n = 51$.

and then cancer cell proliferation, survival, invasivity, and metastasis (Lee et al. 2012; Paradkar et al. 2014). Other publications reported a significant relationship between dysregulation of expression of some cytokines (e.g. LCN2, IL-1, IL-2, IL-4, IL-6, TNFα, and etc.) and incidence of cervical pre-cancerous conditions, as well as cellular progression to cancer, cancer invasivity, and metastases (Syrjänen et al. 2010; Ali et al. 2012; Paradkar et al. 2014). A potential linkage of these events is based on a fact that while immuno-stimulatory T-helper (T_H) cell Type 1 cytokines like TNFα, IFNγ, IL-2, and IL-12 can induce cell-mediated immunity and tumor suppression, they can also have a pro-inflammatory role in a host. In contrast, inhibitory T_H2-type cytokines (e.g. IL-4, -5, -6, -8, -10) reduce cell-mediated immunity and concurrently induce humoral immunity. As such, the above-noted facts suggest that cytokine-related immune responses in the process of cancer are complex and heterogeneous.

There are limited numbers of studies on inflammation markers in HPV^+ or CC patients. Most of those studies were focused on a narrow spectrum of cytokines (Nguyen et al. 2005; Lieberman et al. 2008; Syrjänen et al. 2010; Ali et al. 2012; Scott et al. 2013) few studies have performed analyses with large cytokine panels (Lieberman et al. 2008). Further, even less have reported on systemic cytokines levels (Ali et al. 2012) in test subjects. The present study assessed a spectrum of cytokines as indicators of possible systemic inflammation in CC patients. The data herein revealed a significant increase in serum TNFα, IFNβ, IL-1β, TREM-1, and IL-6 levels in CC patients compared to in NILM controls. The increases noted in systemic levels of pro-inflammatory TNFα and IL-1β as well as in sTREM-1 is consistent with a theory that attempts to explain the role of immune system components in neoplastic processes (Paradkar et al. 2014; Todoric et al. 2016).

Activated macrophages secrete TNFα principally in response to acute inflammation. This helps to explain why circulating levels of TNFα are increased during fever, sepsis, cancers, Alzheimer's disease, and irritable bowel syndrome (Scott et al. 2013). In the current study it was seen that systemic TNFα levels were significantly up-regulated in the CC patients compared to in NILM controls. This outcome is in line with that of increased TNFα levels in local tissue specimens from CC patients (Lieberman et al. 2008). Of note, Scott et al. (2013) suggested that significant increases in levels of systemic TNFα were associated with a reduced likelihood of HPV clearance (including low and high-risk types) among women with incident HPV infections, but without cervical intraepithelial neoplasia.

Lieberman et al. (2008) reported only a clear trend of the association of depressed levels of IL-1β in women with incident HPV infection, compared to levels in women with no infection. These authors noted a similar trend in women with persistent HPV infection compared to those with no infection. High levels of IL-1β, IL-6, and IL-8 – but low or undetectable levels of other cytokines – were detected in vaginal washes from patients with and those without cervical cancer in analyses performed using a multiplex assay (Nguyen et al. 2005).

The most novel finding of the current study was a significant increase in serum sTREM-1 in the CC patients. It is unfortunate for now that this data cannot be compared to other studies as there is to date a lack of TREM-1-related studies. Only one single study has been done using patients with invasive cervical cancer and precursor lesions and increased expression of TREM-1 in monocytes (but not in sera) from patients with advanced cancer (Anaya-Prado et al. 2015). Despite existing differences between the study specimen types, those findings support those seen in this current study. TREM-1 is a novel biomarker discovered in 2000. It is an immunoglobulin family member that can be found on the surface of neutrophils, monocytes, macrophages and endothelial cells, and has a role in response to infection. It was discovered that TREM-1 activates the inflammatory reaction, synthesis of inflammatory mediators, and inhibition of anti-inflammatory mediators TREM-1 enables the synthesis of pro-inflammatory cytokines via Toll-like receptor (TLR) and modulates the innate inflammatory response by enhancing the signal pathway mediated by TLR. Thus, increased levels of sTREM-1 are consistent with the presence of systemic inflammation in CC patients.

Increased levels of IFNβ and IL-6 do not argue against the hypothesis about the presence of pro-inflammatory mechanisms underlying CC development. In contrast to the present findings, Lieberman et al. (2008) reported non-significant trends toward lower systemic cytokine levels in hosts with incident and persistent human papillomavirus infection. It is important to keep in mind that IFNβ and IL-6 have multiple roles in host immune responses; as such, question about their actual changes and roles (if any) in CC remain to be clarified. The most striking finding in the Lieberman study was an association between reduced systemic levels of IFNβ, IL-1β, IL-6, and IL-10 and cigarette smoking. Contrary to expectations, no smoking-dependent cytokine alterations were detected in the patients in the present study. This absence of smoking-dependent cytokine alterations might be an important limitation of the current study.

In summary, the finding here of increased serum TNFα, IFNβ, IL-1β, sTREM-1, and IL-6 in the CC subjects is suggestive of some important roles for each in pathophysiology of CC. The observed changes in expression are most likely CC-related and provide support for a hypothesis of systemic inflammation being involved in CC. Nevertheless, additional studies are required to clarify the importance of these cytokine alterations in CC patients.

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Declaration of interest

The authors declare no conflict of interest. The authors alone are responsible for the content of this manuscript.

Author contributions

Study conception and design: VD, NRJ; data acquisition: VA, CJ, JK, VD; analysis and interpretation of data: VA, UD, SE; drafting of manuscript: VA, DU, SE; critical revision: VD.

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