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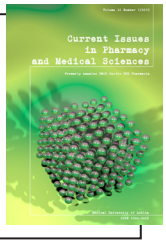

Comparison of TGF-β, IL-10 levels and LMP-1 in gastric and oropharyngeal carcinoma associated with EBV infection

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ABSTRACT

Increasing interest has been focused on the Epstein-Barr Virus (EBV)-associated cancers, including oropharyngeal cancer (OPC) and gastric cancer (GC). Different cytokines, growth factors and proteins take part in oncogenesis. The aim of our study was to generate a comparison of interleukin 10 (IL-10) and transforming growth factor β (TGF-β) levels, as well as latent membrane protein (LMP-1), Epstein-Barr virus capsid antigen (EBVCA), Epstein-Barr virus nuclear antigen (EBNA) and early antigen (EA) frequency in the serum of patients with GC and OPC. The study involved 50 patients with diagnosed GC and 50 patients with OPC. All studied patients were EBV positive. Fresh-frozen tumor tissue fragments were tested using nested PCR assay for EBV DNA detection. Sera from all individuals were investigated using ELISA tests to detect the presence of EBVCA IgG, EBNA IgG, EA IgG, as well as to determine the levels of IL-10 and TGF-β. The obtained results were subjected to statistical analysis. In patients with GC, the levels of TGF-β and IL-10 were significantly higher than in OPC patients. However, the frequency and level of EBVCA, EBNA and EA in patients with OPC and GC were not significantly different. In contrast, TGF-β and IL-10 levels were significantly higher in EBVaGC, as compared to OPC, suggesting their role in gastric carcinogenesis. The differences in frequency of LMP-1 detection in patients with OPC and GC may suggest different mechanism of oncogenesis. Further studies are required to clarify the role of Epstein-Barr virus in cancer development.

INTRODUCTION

Epstein-Barr virus (EBV) is a widespread virus belonging to the Herpesviridae family, which infects more than 90% of the human population worldwide [1,2]. It is also the first human virus with attributable oncogenic potential [3], so there is still increasing number of researches concerning cancers associated with EBV infection, e.g. gastric cancer (GC) and oropharyngeal cancer (OPC).

Nowadays, more than 950,000 cases of gastric adenocarcinoma are diagnosed in the world annually and the relationship between this type of cancer and EBV is estimated to be around 10%. A meta-analysis comprising 9,738 cases evaluated the frequency of EBV-associated gastric cancer (EBVaGC) at the level of 8.8% [4]. Every year, EBVaGC is diagnosed in 84,000 persons and just in the year 2012, 723,000 deaths were recorded due to this malignancy. In Poland, in 2012, EBVaGC accounted for 12.5% of all registered cases of GC [5].

Head and neck cancer (HNC) is the sixth leading cancer in the world, with more than 600,000 new cases reported each year [2]. Squamous cell carcinoma (SCC), the most frequently diagnosed cancer in the head and neck region, is a very important global health problem [2].

In our previous researches, EBV DNA and a high level of antibodies, particularly anti-early antigen (EA) were very frequently detected in Polish patients with GC and OPC [6].

Different cytokines and growth factors take part in oncogenesis. Many studies indicate that transforming growth factor β (TGF-β) induces EBV reactivity in certain types of cancer [7]. Latent membrane protein 1 (LMP-1) is a well-known oncoprotein taking part in neoplastic

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transformation of EBV-infected lymphocytes B and epithelial cells [8].

The aim of our study was the comparison of interleukin 10 (IL-10) and TGF-β levels, as well as LMP-1. Moreover, serum frequency and level of antibodies against Epstein-Barr virus capsid antigen (anti-EBVCA), Epstein-Barr virus nuclear antigen (anti-EBNA) and early antigen (anti-EA) of patients with GC and OPC were compared.

MATERIAL AND METHODS

Patients

The study consisted of a group of 50 patients with diagnosed gastric cancer who were hospitalized at the Surgery Ward of Private Hospital in Nałęczów and AMG Hospital Center in Ryki (Poland) in the years 2012- 2016. All patients were *Helicobacter pylori* negative. The second group involved 50 patients with diagnosed and histopathologically confirmed oropharyngeal squamous cell carcinoma hospitalized at the Otolaryngology Division of the Hospital in Radom, Poland. All patients had neither previous radiotherapy nor chemotherapy. All studied patients, both with GC and OPC, were EBV DNA positive.

The research material consisted of the sera and fresh frozen tumor tissue fragments. This research was approved by the Medical University of Lublin Ethics Committee and is in accordance with the GCP regulations (no. KE-0254/133/2013).

DNA extraction from fresh frozen tumour tissue; detection of EBV DNA

DNA extraction from fresh frozen tumour tissue, detection of EBV DNA, amplification of EBNA-2 gene (the nested PCR) and genotyping of LMP-1 were performed as previously described [6].

Serological tests

To detect antibody levels, serological tests were used with ELISA method. Designed antibodies: anti-VCA IgM (NovaLisa Epstein-Barr Virus VCA IgM/Nova Tec Immunodiagnostica GmbH/Germany/catalog number: EBVM0150), anti-VCA IgG (NovaLisa Epstein-Barr Virus VCA IgG/Nova Tec Immunodiagnostica GmbH/Germany/catalog number: EBVG0150), and anti-EBNA IgG (NovaLisa Epstein-Barr Virus EBNA IgG/Nova Tec Immunodiagnostica GmbH/Germany/catalog number: EBVG0580), antibodies anti-EA IgG (ELISA-VIDITEST anti-EA (D) EBV IgG/Vidia/Czech Republic/catalog number: ODZ-006). All tests were performed according to the manufacturer's instructions.

The NovaTec Epstein-Barr Virus (EBV) IgG-ELISA is intended for the qualitative determination of IgG class antibodies against Epstein-Barr virus. Samples are considered positive if the absorbance value is higher than 10% over the cut-off. The level of antibodies is expressed as NovaTec-Units=NTU.

ELISA-VIDITEST anti-EA is a semi-quantitative test. Samples with absorbances higher than 110% of the cut-off value are considered positive.

Measuring of cytokines level

The levels of Il-10 and TGFβ were established in the sera of patients by ELISA, using commercially available kits (Diaclone SAS, France). The cytokine level of each serum sample was tested three times and the results are the means of these. The level of tested cytokines is expressed in pg/ml or ng/ml. The minimum detectable dose of IL-10 is less than 0.98 pg/ml (IL-10 HS ELISA kit) cat. no. 850.880.096; TGFβ – 8.6 pg/ml cat. no. 650.010.096.

Statistical analysis

Descriptive statistics were used to characterize patient baseline characteristics. The Mann Whitney-U test was applied to compare the antibody, TGFβ and IL-10 levels. Pearson's chi-square test was used to investigate the relationship between LMP-1 subtype and clinical and demographical parameters. Statistical significance was defined as $p < 0.05$.

RESULTS

There were no statistically significant differences between the patients of both groups regarding sex, age, place of residence, tobacco smoking and alcohol consumption, and, therefore, the features did not affect the values of examined parameters. The socio-demographic and clinical-pathological characteristics of the study group of patients are shown in Table 1.

Table 1. Socio-demographic characteristics of patients with OPC and GC

Parameters		OPC patients N=50		GC patients N=50		p
		N	%	N	%	
Sex	Male	29	58.0	31	62.0	0.6831
	Female	21	42.0	19	38.0	
Age	40-59	20	40.0	15	30.0	0.2945
	60+	30	60.0	35	70.0	
Place of residence	Urban	20	40.0	11	22.0	0.0517
	Rural	30	60.0	39	78.0	
Tobacco smoking	Yes	32	64.0	37	74.0	0.2797
	No	18	36.0	13	26.0	
Alcohol abuse	Yes	30	60.0	35	70.0	0.2945
	No	20	40.0	15	30.0	

Pearson's chi-square test

OPC patients – oropharyngeal cancer patients
GC patients – gastric cancer patients

Accordingly, 78% of all patients with GC and 60% with OPC came from rural areas. The majority of cases, both with GC and OPC, smoked tobacco (74% and 64%, respectively) (Table 1).

In patients with EBVaGC, the levels of TGF-β and IL-10 were significantly higher than in OPC patients (Table 2).

The patients infected with wild type of EBV were predominant in OPC-patients (78%), whereas in 20% of GC cases, LMP-1 was not detected (Table 3). The frequency and level of antibody against EBVCA, EBNA and EA in patients with OPC and GC were not statistically different (p>0,05).

Table 2. Serum level of IL-10, TGF-β and EBV antibodies in patients with OPC and GC

Parameters	OPC Mean ± SD	GC Mean ± SD	p
IL-10 (pg/ml)	2.3±1.5	7.2±3.1	0.0003*
TGF β (ng/ml)	11.3±5.6	24.4±6.8	0.0005*
anti-EBVCA (U)	68,3±5.3	67.4±5.3	>0.05
anti-EBNA (U)	72,4±21.1	72.3±17.7	>0.05

* statistically significant (Mann Whitney-U test)

IL-10 – interleukin 10, TGF β – transforming growth factor β, anti-EBVCA – antibodies against Epstein-Barr virus capsid antigen, anti-EBNA – antibodies against Epstein-Barr virus nuclear antigen

Table 3. Frequency of LMP-1 and EBV antibodies in patients with OPC and GC (%)

		OPC	GC	p
		N= 50	N= 50	
EBV type	wt-LMP-1	78.0	64.0	1×10^{-5}*
	del-LMP-1	22.0	16.0	
	LMP-1 not detected	0	20.0	
Antibodies Positive (%)	anti-EBVCA IgG	88.0	95.0	0.1404
	anti-EBNA IgG	90.0	93.0	0.7124
	anti-EA IgG	72.0	75.0	0.6484

* statistically significant (Pearson's chi-square test)

wt-LMP-1 – wild type LMP-1; del-LMP-1 – LMP-1 type with deletion, anti-EBVCA – antibodies against Epstein-Barr virus capsid antigen, anti-EBNA – antibodies against Epstein-Barr virus nuclear antigen, anti-EA – antibodies against early antigen

DISCUSSION

According to the epidemiological studies, about 90% of the global population is infected with EBV, however, only some individuals develop EBV-associated cancers [9]. Primary infection with EBV is followed by a latent phase in B lymphocytes, with periods of reactivation and transmission of virus in the oropharyngeal epithelium. Chronic viral infection is a significant risk factor for both OPC [1] and GC [9]. The ability to enter the latency is a very important feature of EBV, as only some genes are expressed in this phase [10].

EBVaGC exhibits type I or type II latency [10], in which apart from EBNA-1, EBERs, BARTs, BART miRNAs, also LMP-1 and LMP-2 are expressed. Epstein-Barr nuclear protein 1 (EBNA-1) is present in all latency types, and also EBNA is the only type of protein produced during activation into the lytic cycle. Epstein-Barr Nuclear Protein-2 (EBNA-2) is the major transcription factor that regulates the expression of viral genes and activates LMP-1 and LMP-2 promoters. It can also transactivate many cellular genes crucial in cell proliferation and immortalization following the infection.

In our study, in fresh frozen tissue sections with GC, LMP-1 was detected in 80.0% of all cases. The wild type EBV was detected in a majority of cases (64.0%), while deletion in the LMP-1 was found in 16.0%. However, LMP-1 was not detected in 20% of all GC patients, while, LMP-1 was detected in all patients with OPC – wt-LMP-1 in 78% and del-LMP-1 in 22% of all cases. The differences in frequency of del-LMP-1 in patients with OPC and GC may suggest different mechanism of oncogenesis.

According to Wang *et al*. [10], EBVaGC shows type II latency, and due to it, LMP-1 is not expressed. In the study performed by these researchers, this oncoprotein was detected only in 1 case among 30 patients. The authors state that LMP-1 is expressed more frequently in nasopharyngeal cancer (NPC).

Other studies have reported differential expression and occurrence of this protein in NPC and EBVaGC. In some researches, expression of LMP-1 in the primary EBVaGC was low, while in others it was high [8]. EBV with a deletion in LMP-1 is more common in nasopharyngeal cancer [10]. In research carried out by Neves *et al*. [8], wild-type LMP-1 (wt-LPM-1) in nasopharyngeal carcinoma was detected in 94.9% of all patients and only in 28.6% of the healthy control group. However, in Japanese patients with gastric cancer, the frequency of del-LMP-1 was similar both in the cases and control groups (91.7% vs. 83.3%). The authors state, therefore, that LMP-1 type with deletion (del-LMP-1) is more common in the Japanese population [11]. Some researchers suggest that due to differences in the latency of EBV infection in EBVaGC and NPC, oncogenic mechanisms of EBV in EBVaGC and NPC may be different [10].

Tsao *et al*. [12] imply that the role of LMP-1 may be various at different stages of carcinogenesis. High expression of LMP-1 can cause deregulation of tumour cells, a cytotoxic effect, and may lead to increased rates of mutation and methylation of EBVaGC.

Virus reactivation and entering into the lytic cycle may be caused by different factors, e.g. decrease in host resistance, exposure to some chemicals, TGF-β [13]. In massively infected EBVs, small EBVs encode small RNAs [14-15]. EBER induces signals and through TLR9, stimulates the production of pro-inflammatory cytokines [17], while, LMP-1, by B-cell activation, increases the production of interleukin [18]. Various cytokines, such as IL-10 and TGF-β, play a crucial role in the process of tumour development [19,20]. Our previous results revealed significantly higher levels of IL-10 and TGF-β in serum of patients with oropharyngeal cancer [6] and with gastric cancer [21] than in the control group.

BCRF1 encodes viral IL-10 (vil-10), which has a high homology with human IL-10 [18,19]. It is secreted in the later stages of the lytic phase and also at the beginning of B lymphocytes infection. Some researches revealed that elevated serum levels of IL-10, in fact, were observed in patients with advanced gastrointestinal malignancies, when compared with healthy control and may be helpful in the assessment of disease progression [20].

Disorders in the synthesis and function of TGF-β and its signaling pathways are found in many pathological states, including tumors. Signaling pathways in tumors induced by these ligands can lead to the inhibition of carcinogenesis or progression of cancer. It seems that, with regard to this, the most important role in cancer transformation is involvement in the invasion and migration of tumor cells and immunosuppression. TGF-β secreted by tumor cells decreases the immune response of the body and causes changes in the phenotype of macrophages and neutrophils. Therein, macrophages are converted into tumor-associated macrophages (TAMs) [7,13]. Our research revealed higher

levels of TGF-β in serum of patients with EBVaGC (24.4 ng/ml), compared to the OPC group (11.3 ng/ml) ($p = 0.0005$). According to the medical history of the patients, it seems that *H. pylori* was not detected in anyone. The limitation of our study is the small number of patients, which makes detailed epidemiological analysis impossible.

A study performed by Shukla *et al*. [13] among 95 patients with EBVaGC, expression of TGF-β mRNA was revealed in 89.5% of all cases, which may indicate the role of TGF-β in the development of gastric cancer. Moreover, the researchers found that the expression was closely related to the lytic phase of infection in patients without *H. pylori*, and, therefore, they suggest that TGF-β may influence the reactivation of EBV. As we showed previously, the concentration of TGF-β correlated with the high anti-EA antibody titer [21].

CONCLUSIONS

Our results show that the levels of TGF-β and IL-10 were significantly higher in EBVaGC, as compared to OPC, suggesting their role in gastric carcinogenesis. LMP-1 was detected in all patients with OPC: wt-LMP-1 in 78% and del-LMP-1 in 22% of all cases. The differences in frequency of LMP-1 detection in patients with OPC and GC may suggest different mechanisms of oncogenesis. These data are important in the future studies because the knowledge of the specific mechanisms of GC and OPC development could be helpful for future diagnostics and therapy strategies. However, further studies should be carried out to clarify the role of Epstein-Barr virus in cancer development, as genetic and epigenetic changes occur after EBV infection.

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