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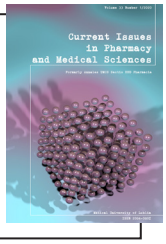

The role of toll-like receptor 9 (TLR9) in Epstein-Barr virus-associated gastric cancer

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ABSTRACT

Epstein-Barr virus-associated gastric carcinoma (EBVaGC) is the most common malignancy caused by EBV infection. Toll-like receptors (TLRs) as major components of innate immune system are crucial in the development of inflammatory processes and carcinogenesis. The aim of our study was to evaluate tissue and serum level of TLR9 in EBV-positive and EBV-negative gastric cancer patients. The study involved 30 EBV(+) and 30 EBV(-) patients. EBV DNA was detected in fresh frozen tumor tissue. In serum samples TLR9 level, transforming growth factor β (TGFβ), interleukin 10 (IL-10) and antibodies against EBV were detected using ELISA tests. TLR9 level was also measured in homogenate of tumour tissue. TLR9 level was statistically lower in EBV(+) patients both in serum and tissue, with statistically higher level in tissue than in serum. Lower level of TLR9 was accompanied by higher level of Epstein-Barr virus capsid antigen (EBVCA), Epstein-Barr virus nuclear antigen (EBNA) and early antigen (EA). A lower level of TLR9 was detected in patients with poorly differentiated cancer (G3) and greater lymph nodes involvement (N3-N4). Lower level of TLR9 in patients with EA may point to TLR9 role in reactivation of EBV infection.

INTRODUCTION

Gastric cancer (GC) as the fifth most frequent cancer in the world, was responsible for over 1,000,000 new cases in 2018 and an estimated 783,000 deaths worldwide [1]. In Poland, in the year 2015, registered frequency of deaths due to GC among males was 6.1% (more than 3,400 deaths) and 4.1% (1,860 deaths) among females [2].

Epstein-Barr virus (EBV), (human herpes virus 4; HHV-4), belonging to the *Herpesviridae* family is wide-spread – infecting over 90% of the world's population. EBV establishes lifelong, latent infections and has the ability to cause chronic infections [3-6]. Many authors describe an association between chronic EBV infection and development of different human malignancies such as nasopharyngeal carcinoma (NPC), Hodgkin's lymphoma, extranodal NK/T-cell lymphoma, as well as gastric carcinoma [7,8].

According to The Cancer Genome Atlas (TCGA), GC is divided into four subtypes including, among others, EBV-positive tumors [9]. Numerous studies have focused on the relationship between host cellular immune responses and EBV associated gastric cancer (EBVaGC) prognosis [10]. Congenital immunity is the body`s first line of defense against infection. Its effect is determined by pattern recognition receptors (PRR) that possess the ability to recognize different structures of microorganisms called 'pathogen associated molecular patterns' (PAMP). Thanks to PRR, the human immunological system distinguishes foreign antigens from its own ones. PRR representatives include Toll-like receptors (TLR), which play the major role in recognizing the infection and triggering the immunological response [11]. Various researches revealed the role of TLR9 in congenital immunity to infection with DNA viruses, such as Herpes simplex viruses (HSV) and EBV [12,13]. Our previous study showed that the level of antibodies against EBV such as EBV capsid antigen (EBVCA), EBV nuclear antigen (EBNA) and early antigen (EA) was significantly higher in patients with GC than in a control group, which points to EBV reactivation. Transforming growth factor beta

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(TGFβ) level was significantly higher in EBVaGC patients – suggesting its role in gastric carcinogenesis [14].

Therefore, the aim of our study was to evaluate the tissue and serum level of TLR9 in EBV-positive and EBV-negative GC patients. The tissue and serum level of TLR9 was also analyzed regarding histological grading and TNM classification. In addition, the correlation between serum and tissue level of TLR9 and serum level of EBVCA, EBNA, EA and TGFβ was determined.

MATERIALS AND METHOD

Patients

The present study involved 60 patients with diagnosed and histopathologically confirmed GC who were hospitalized at the surgery wards of hospitals in the Lublin region, Poland. This group consisted of 30 EBV-positive and 30 EBV-negative patients. All patients were *Helicobacter pylori* negative (based on the medical history of the patient).

The study was approved by the Medical University of Lublin Ethics Committee, and is in accordance with the GCP (Good Clinical Practice) regulations (No. KE-0254/135/2017, 25 May 2017). Informed written consent was collected from all participants.

Clinical specimens

The tissue samples were collected from all patients during surgery and frozen at -80°C until analysis. TNM classification was determined during primary diagnosis according to Union for International Cancer Control (UICC) and American Joint Committee on Cancer (AJCC) system (7th edition) standards [15]. All tumor samples were histologically examined and classified according to WHO criteria [16].

Serum collection

Venous blood samples from all patients were centrifuged at 1500 rpm at room temperature for 15 min, following which the serum was collected and frozen at -80°C until its analysis.

Molecular methods

EBV DNA detection and the amplification of the Epstein-Barr nuclear antigen 2 (EBNA-2) gene (the nested PCR) were performed as previously described [17]. The nested PCR was carried out for amplification of Epstein-Barr nuclear antigen 2 (EBNA-2). The sequence of primers used for PCR was as follows: outer pair 5' – TTT CAC CAA TAC ATG ACC C – 3', 5' – TGG CAA AGT GCT GAG AGC AA – 3' and inner pair 5' – CAA TAC ATG AAC CRG AGT CC – 3', 5' – AAG TGC TGA GAG CAA GGC MC – 3'.

All PCR reactions were carried out in the final volume of 25 µl, using HotStartTaq DNA Polymerase (Qiagen, Germany). Concentrations of PCR reaction components were prepared as follows: 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each forward and reverse primers and 0.5 U of HotStartTaq polymerase. During each run the samples were tested together with one negative (nuclease-free water) and positive control (EBV-positive cell line, Namalwa, ATCC-CRL-1432).

Serological methods

In the collected serum samples, serological tests for antibodies against EBV were conducted by means of the immunoenzymatic ELISA method, using the commercially available ELISA tests as previously described [17].

Designed antibodies include: anti-VCA IgM (Nova-Lisa 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), anti-bodies anti-EA IgG (ELISA-VIDITEST anti-EA (D) EBVIgG/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 semiquantitative test. Samples with absorbances higher than 110% of the cut-off value are considered positive.

TLR9 assay

Serum and tissue level of TLR9 was marked with a Cloud-Clone Corp. USA (HEA709Hu) kit according to the manufacturer's instruction. The kit is a sandwich enzyme immunoassay for *in vitro* quantitative measurement of TLR9 in human serum, plasma, tissue homogenates, cell lysates, cell culture supernates and other biological fluids. The concentration of High Sensitive Toll Like Receptor 9 (TLR9) in the samples is then determined by comparing the O.D. of the samples to the standard curve. Absorbance was measured in a spectrophotometer Epoch (Biotek Instruments, USA). The results were analyzed with the use of software for the reader Gen 5 (Biotek Instruments, USA). The serum level of TLR9 is presented in pg/ml. The minimum detectable dose of this kit is typically less than 12.1 pg/ml.

TLRs concentrations in tissue homogenates were adjusted to total protein level and were expressed as pg/g of protein. To determine TLRs protein concentration, tissue samples were rinsed with 0.9% NaCl and stored at -80°C until time of analysis. Tissue homogenates (10% w/v) were prepared in 0.1 mol. l–1Tris-HCl buffer, pH = 7.4 using a laboratory MPW-120 homogenizer, and supernatants were obtained by centrifugation at 5000 × g for 30 min.

Protein was measured using the method of Bradford (1976). The assays were performed with the use of spectrophotometer SPECORD M40 (Carl Zeiss, Jena, Germany).

Statistical analysis

Descriptive statistics were used to characterize patient baseline characteristics. Pearson's chi-square test was applied to investigate the relationship between clinical and demographical parameters. The Mann-Whitney U-test was employed to compare TLR9, antibodies and cytokine level. The correlation between TLR9 level and

the examined parameters was assessed through application of the Spearman correlation rank test. Statistical significance was defined as $p<0.05$.

RESULTS

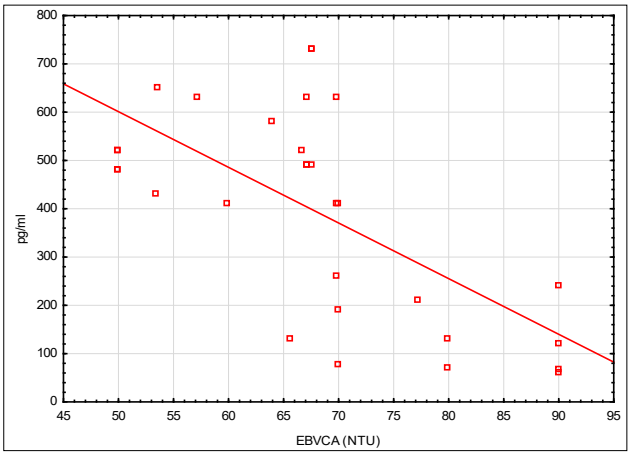
Clinical and epidemiological characteristics of the patients are presented in Table 1. There were no statistically significant differences between either group of patients.

Table 1. Clinical and epidemiological characteristic of gastric cancer patients

		EBV				p
		Positive		Negative		
		n	%	n	%	
Sex	Female	19	63.3	19	63.3	>0.05
	Male	11	36.7	11	36.7	
Age	50-69	5	16.7	5	16.7	>0.05
	70+	25	83.3	25	83.3	
Place of residence	Urban	7	23.3	6	23.3	>0.05
	Rural	23	76.7	24	76.7	
Smoking	Yes	21	70.0	21	70.0	>0.05
	No	9	30.0	9	30.0	
Alcohol abuse	Yes	18	60.0	18	60.0	>0.05
	No	12	40.0	12	40.0	
G	G1	2	6.7	2	6.7	>0.05
	G2	19	63.3	19	63.3	
	G3	9	30.0	9	30.0	
T	T1-T2	21	70.0	21	70.0	>0.05
	T3-T4	9	30.0	9	30.0	
N	N1-N2	18	60.0	18	60.0	>0.05
	N3-N4	12	40.0	12	40.0	
M	M0	30	100.0	0	100.0	-

n - number of patients; G – grading; T – tumor size; N – lymph nodes; M – metastasis

The present study revealed that in EBV-positive patients, the level of TLR9 (in serum and tissue) was significantly lower, while EBVCA, EBNA, TGFβ, as well as Il-10 level was statistically higher than in the group of EBV-negative patients (Table 2). The level of TLR9 in tissue was significantly higher than in serum.



Spearman correlation rank test (EBVCA&TLR9, r=-0.631462; p=0.0001)

Figure 1a. Correlation between serum level of TLR9 and serum level of EBVCA

Table 2. Level of TLR9, EBVCA, EBNA, TGFβ and IL-10 in EBV-positive and EBV-negative gastric cancer patients

	EBV positive	EBV negative	p
	±SD	±SD	
TLR9 serum	392.9±215.2	961.9±232.1	0.0001*
TLR9 tissue	963.2±627.7	1985.9±369.5	0.0001*
EBVCA	67.4±5.3	37.4±22.3	0.0001*
EBNA	72.3±17.7	48.2±14.8	0.0001*
TGFβ	25.4±6.8	12.2±7.2	0.0001*
IL-10	7.1±2.7	4.5±0.9	0.00006*

*statistically significant; Mann Whitney U-Test

TLR9 – Toll-like receptor 9; EBVCA – Epstein-Barr virus capsid antigen; EBNA – Epstein-Barr virus nuclear antigen; TGFβ – transforming growth factor β; IL-10 – interleukin 10

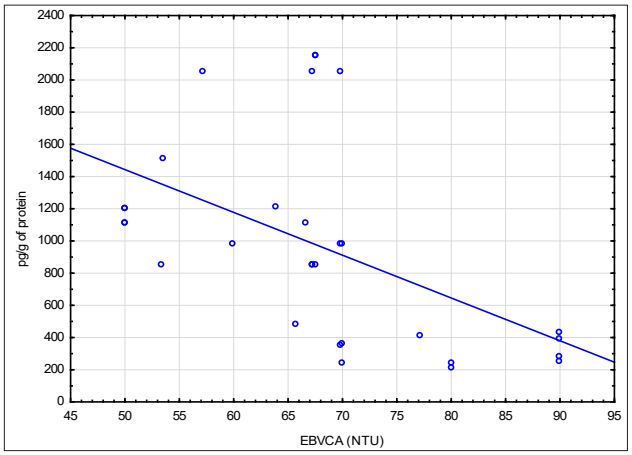
Differences were found in TLR9 concentration, depending on the degree of tumor differentiation (G). In patients with G2-G3, TLR9 level was lower both in tissue (p=0.0276) and in serum (p=0.0413). No relation was found between the level of TLR9 and tumor dimension (T), whereas in patients with lymph nodes involvement N3-N4, the level of TLR9 in tissue was statistically lower than in N1-N2 (p=0.0211) (Table 3).

Table 3. Tissue and serum level of TLR9 according to GTN

	EBV(+)		EBV(-)	
	TLR9 in tissue ±SD	**TLR9 in serum** ±SD	**TLR9 in tissue** ±SD	**TLR9 in serum** ±SD
G1	3245.1±7.0	414.2±20.6	2121.3±43.8	975.8±20.8
G2-G3	1014.5±61.8	95.2±4.9	1933.2±33.8	956.0±24.6
P	0.0276*	0.0413*	0.1725	0.7388
T1-T2	983.5±66.5	395.8±18.9	2120.4±43.8	976.5±20.7
T3-T4	915.8±56.5	391.8±23.0	1923.4±32.7	957.4±24.6
P	0.8563	1.0	0.1715	0.7787
N1-N2	1251.5±52.3	490.4±13.8	2076.3±40.8	991.1±30.9
N3-N4	771±63.1	328.0±23.6	1914.9±33.4	939.0±15.8
p	0.0211*	0.0789	0.3512	0.7629

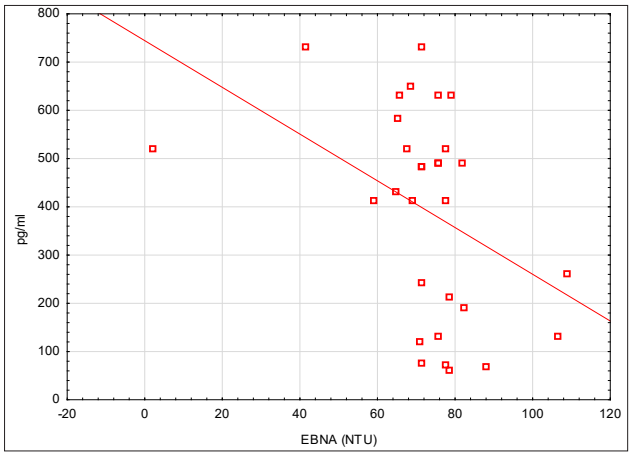
*statistically significant; Mann Whitney U-Test

A correlation was stated between TLR9 level (both in tissue and serum) and anti-EBVCA, EBNA and EA antibodies level (Fig. 1-3). An increase in the antibody titer was accompanied by a decrease of TLR9 level.



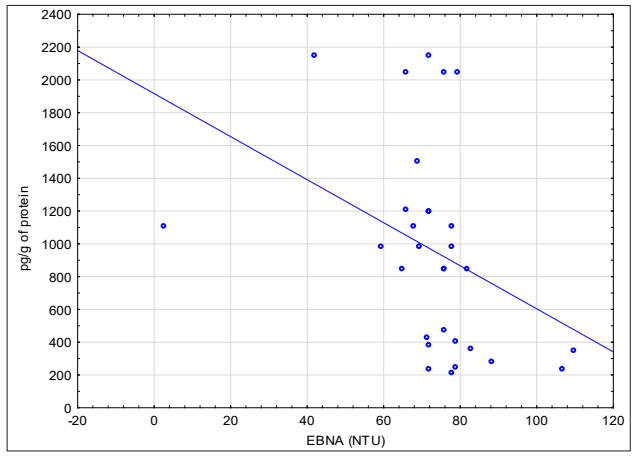
Spearman correlation rank test (EBVCA&TLR9, r=-0.642650; p=0.0001)

Figure 1b. Correlation between tissue level of TLR9 and serum level of EBVCA



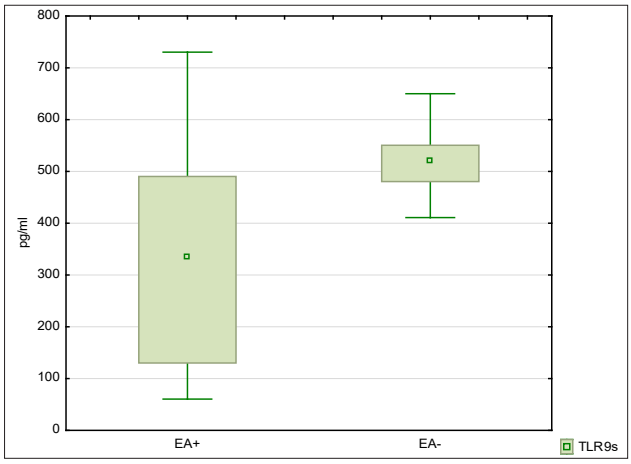
Spearman correlation rank test (EBNA&TLR9, r=-0.4132; p=0.02323)

Figure 2a. Correlation between serum level of TLR9 and serum level of EBNA



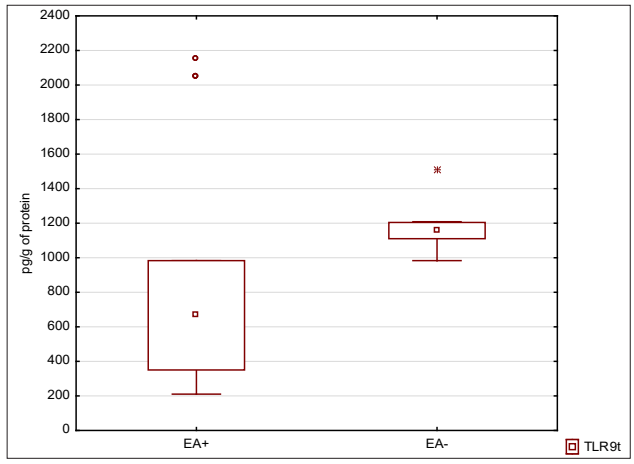
Spearman correlation rank test (EBNA& TLR9, r=-0.506471; p=0.0043)

Figure 2b. Correlation between tissue level of TLR9 and serum level of EBNA



Mann Whitney U-Test; (p=0.0639)

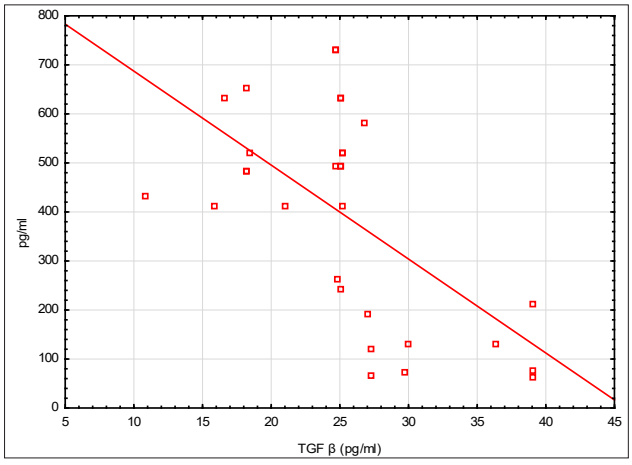
Figure 3a. Serum level of TLR9 and presence of anti-EA



Mann Whitney U-Test; (p=0.0292)

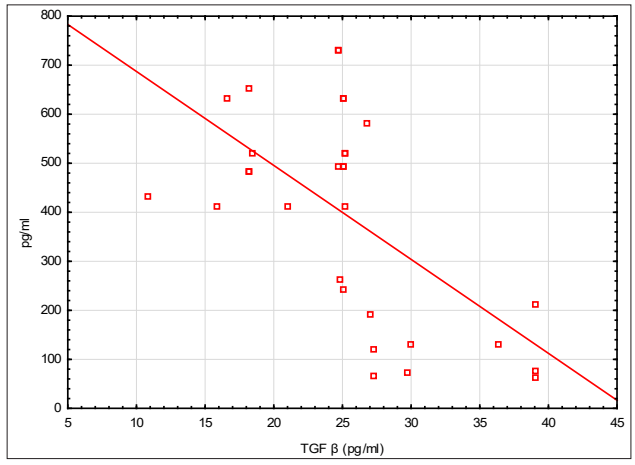
Figure 3b. Tissue level of TLR9 and presence of anti-EA

A similar relationship was observed for TGFβ (Figure 4). Increase of TGFβ level co-occurred with decrease of TLR9 level (both in serum and tissue)



Spearman correlation rank test (TGFβ&TLR, r=-0.613785; p=0.0003)

Figure 4a. Correlation between serum level of TLR9 and TGFβ



Spearman correlation rank test (TGFβ&TLR9, r=-0.6312; p=0.0002)

Figure 4b. Correlation between tissue level of TLR9 and TGFβ

DISCUSSION

According to recent theory, infection is one of the major factors responsible for the development and progression of cancer [18]. The causal mechanism of this phenomenon is not clarified despite the fact that the processes taking place at the site of infection are well understood. A lot of anti-inflammatory cytokines and growth factors, including TGF-β, released in the final stage of the infection, migrate to epithelial cells [19].

One of the mechanisms in oncogenesis is the interaction between highly conservative fragments of the genome with different pathogens, including viruses, and TLRs. A lot of data point to the role of TLRs in the development of the inflammatory process and the growth of cancer [20]. However, TLRs can play a stimulating role on the one hand, while on the other – they may inhibit the development of cancer. Recent studies point out that they can be activators of anti-cancer immunity or may stimulate progression of malignancy [19].

In infected cells, EBV codes small RNA (EBER) [21]. EBER induces signals and through TLR9, it stimulates the production of interferon and cytokines [22]. Our previous studies concerning patients with GC [14] revealed that the level of TGF-β and IL-10 in serum was statistically higher in patients with high level of anti-EA antibodies and can point to reactivation of EBV infection in patients with GC. The present study showed statistically higher level of TGFβ in the group of EBV-positive patients, as compared to the EBV-negative group. This result can confirm that TGF-β plays a role in EBV reactivation. Of note, some authors suggest EBV reactivation occurs at the early stages of oncogenesis [23,24].

TGFβ secreted by cancer cells silences the immunological response and causes changes in the phenotype of neutrophils and macrophages, which are, in turn, transformed into macrophages characteristic of cancers – tumor-associated macrophages (TAM) [25]. Similarly, a significantly higher level of IL-10 was found in EBVaGC cases as compared to EBV-negative ones. BCRF1 codes viral IL-10 (vil-10), which shows considerable homology with the human IL-10 [26]. A number of studies reveal that IL-10 concentration is higher in patients with GC, as compared to the controls [27]. It is known that IL-10 plays an important role in modulating the immunological response both in normal conditions and in the cancer microenvironment [28,29].

A number of relationships were described between TLRs polymorphism and infectious diseases, as well as cancer [30]. TLRs polymorphism is associated with a risk of GC development, which suggests that signals are transmitted both on the molecular and genetic level. In gastric dysplasia, a high expression of TLRs occurs, which means that TLRs play important role in the development of this cancer. However, results of studies vary in different parts of the world [31]. In Japan, no connection was found between TLRs polymorphism and *H. pylori* infection, while in Brazil, polymorphism of TLR2, TLR4 and GC was discovered [31].

The present study found that in EBVaGC cases, the level of TLR9 was considerably lower as compared to EBV-negative cases, but it was definitely higher in homogenate from tissue in comparison to serum.

Fernandez-Garcia *et al.* [32] analyzed the expression of TLR3, TLR4 and TLR9 in patients with GC, pointing to their increased expression. Accordingly, TLR3 and TLR9 react with *H. pylori*, and as a consequence, this leads to chronic gastritis. However, the results obtained by these researchers did not indicate any relation between TLR expression and clinical features. The authors suggest that TLR3 expression is related to cancer localization, greater aggressiveness and bad prognosis. Though, TLR9 expression in their work depended neither on histological differentiation of cancer, nor tumor dimensions and lymph nodes involvement. The present study did not reveal any statistically significant relationship between tumor dimension and TLR9 level. On the other hand, a lower level of TLR 9 was detected in patients with poorly differentiated cancer (G3) and greater lymph nodes involvement (N3-N4).

Our research revealed that an increase in the level of EBVCA, EBNA and EA was accompanied by a decreased level of TLR9. Therefore, it can be presumed that the level of TLR9 decreases in reactivation of latent infection to the lytic phase, whereas no relationship was found between the level of TGFβ and TLR9. Other authors also detected higher level of antibodies in EBVaGC patients, including EBVCA [4,33].

GC connected with EBV infection is a heterogeneous cancer in which mutation changes, viral DNA and host DNA play a pivotal role [34]. Due to a very small size of tumour tissue samples available for our research, only the PCR method was used to detect EBV DNA, which may be a limitation of our study. Complementing PCR with immunohistochemical analysis could have provided more conclusive data. Liu *et al*. [6], for example, identified 9 different variants of EBV genomes in biopsy specimens of GC. So differences between the isolates might be a result of co-infection with various genetic variants of EBV.

CONCLUSIONS

TLR9 concentration was statistically lower in EBV-positive than in EBV-negative gastric cancer patients (both in serum and tissue), with the level of TLR9 in tissue statistically higher than in serum

The higher level of EBVCA and EBNA was accompanied by a lower level of TLR9. Moreover, lower level of TLR9 was stated in the presence of EA, which may point to the notion that TLR9 plays a role in the reactivation of EBV infection. Future studies are necessary. It seems that TLR9 may be helpful marker in diagnostic and personalized therapy.

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INFORMED CONSENT

Informed written consent was obtained from all individual participants included in the study.

ETHICAL APPROVAL

The study was approved by the Medical University of Lublin Ethics Committee, and is in accordance with the GCP regulations (No. KE-0254/135/2017, 25 May 2017).

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