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ORIGINAL ARTICLE

Association of interleukin-10, tumor necrosis factor-α and transforming growth factor-β gene polymorphisms with the outcome of diffuse large B-cell lymphomas

Olivera Tarabar · Bojana Cikota-Aleksić · Ljiljana Tukić · Nenad Milanović · Aleksandar Aleksić · Zvonko Magić

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Abstract
Background Published data indicate that common genetic variants in immune/inflammatory response genes can affect the outcome of diffuse large B-cell lymphomas (DLBCL). This study investigated the association of *interleukin* (*IL*)-*10* (*−3575, −1082*), *tumor necrosis factor* (*TNF*)-α *−308* and *transforming growth factor* (*TGF*)-*β Leu10Pro* gene polymorphisms with clinical characteristics and outcome of DLBCL patients treated with rituximab–CHOP therapy.
Methods Between January 2004 and December 2007, a total of 84 patients with newly diagnosed DLBCL entered into this study. Genotypes were determined with PCR-based methodology.
Results Patients presenting with B symptoms had *IL-10 −3575* TA/AA genotypes more frequently than TT genotype [odds ratio (OR) 2.89, 95 % confidence interval (CI) 1.11–7.57; $p = 0.03$]. Carriers of *TGF-β* Pro10 allele more frequently had an advanced clinical stage III/IV (OR 4.65, 95 % CI 1.33–16.19; $p = 0.016$) and intermediate-high/high IPI score (OR 5.37, 95 % CI 1.45–20.0; $p = 0.012$). In rituximab–CHOP-treated patients ($n = 64$), the *TNF-α −308* AG/AA carriers had shorter overall ($p = 0.048$) and event-free survival ($p = 0.07$) compared to GG carriers. In multivariate analysis of prognostic factors for survival, the *TNF-α* AG/AA genotypes were significantly associated with inferior survival of lymphoma patients (OR 0.23, 95 % CI 0.07–0.78; $p = 0.018$).
Conclusion Our results indicate the association of *IL-10 −3575* and *TGF-β Leu10Pro* gene variations with clinical characteristics. In patients treated with rituximab–CHOP therapy, the *TNF-α −308* AG/AA genotypes showed a significantly less favorable survival than the GG genotype.

Keywords Diffuse large B-cell lymphoma · Interleukin-10 · Tumor necrosis factor-α · Transforming growth factor-β · Gene polymorphism

O. Tarabar (✉) · L. Tukić
Clinic of Hematology, Military Medical Academy, Crnotravska 17, 11000 Belgrade, Serbia
e-mail: olivera@tarabar.net

B. Cikota-Aleksić · Z. Magić
Institute for Medical Research, Military Medical Academy, Belgrade, Serbia

N. Milanović
Institute of Oncology and Radiology, Belgrade, Serbia

A. Aleksić
Institute of Occupational Health, Military Medical Academy, Belgrade, Serbia

Introduction

Diffuse large B-cell lymphomas (DLBCL) represent a heterogeneous group of lymphoproliferative disorders with highly variable clinical course and outcome [1]. The addition of rituximab to CHOP (R-CHOP) or CHOP-like chemotherapy has markedly improved the outcome of all subgroups of patients with DLBCL and has established R-CHOP therapy as the standard of care in DLBCL [2–5]. Despite significant advances in immunochemotherapy, response to treatment is heterogeneous, ranging from cure to treatment failure, relapse, and death. This variability in clinical course and outcome is largely due to biological differences between tumors and patients. The etiology of DLBCL remains unknown although there are data that support a role of genetic and immune-related factors in the pathogenesis of lymphoma [6, 7].

The balance of different cytokine signals plays an important role in the regulation of normal immune function. Some evidence indicates that the disturbance of this balance participates in development or evolution of numerous immune-regulated disorders, including lymphoma [8].

Several studies have suggested that cytokine production is influenced by genetic factors [9, 10]. Recently, many single-nucleotide polymorphisms (SNP) were detected within cytokine genes, particularly within their promoter regions [11]. Some of these polymorphisms may be associated with different levels of cytokine expression and also modulate expression of other cytokines involved in the immune response. Numerous studies have provided evidence for the role of interleukin (IL)-10, tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β in the pathogenesis of B-cell lymphomas [12–15]. These cytokines exploit different models to exert their effects in immunoregulation and inflammation. They influence, in a paracrine or autocrine manner, growth and survival of normal and malignant cells, including B lymphocytes [16–18]. These cytokines can also contribute to a drug-resistant phenotype in many tumors [19]. Considering their properties, a number of studies investigated the association between IL-10, TNF-α and TGF-β polymorphisms and lymphoma susceptibility or prognosis [20–27].

The aim of the present study was to investigate the association of genetic variation within *IL-10* (*−3575, −1082*), *TNF-α −308* and *TGF-β Leu10Pro* genes with clinical features at presentation and outcome of DLBCL patients treated with R-CHOP therapy.

Materials and methods

Patients

This study included a total of 84 patients with DLBCL who were diagnosed and treated at the Clinic of Hematology, MMA, Belgrade, Serbia between January 2004 and December 2007. Diagnosis was based on histopathology and immunohistochemistry according to the World Health Organization (WHO) classification [1]. The patients had mandatory baseline examinations that included clinical examination, laboratory tests, chest radiograph, computer tomography of chest and abdomen, and a bone marrow biopsy. The extent of the disease was categorized according to the Ann Arbor classification and the risk score was determined by the International Prognostic Index [28].

Samples of 94 patients were eligible for the study. After excluding patients with previous history of low-grade lymphoma (7 patients), cancer (2 patients), and HIV-related DLBCL (1 patient), the final study population consisted of 84 patients (aged 22–76 years, median 48).

Table 1 Baseline characteristics of patients included in the study

Characteristics	Patients No. (%)	R-CHOP group No. (%)
	84	64
Sex		
Female	41 (48.8)	31 (48.4)
Male	43 (51.2)	33 (51.6)
Age		
≤60 years	57 (67.9)	45 (70.3)
>60 years	27 (32.1)	19 (29.7)
Ann Arbor stage		
I/II	36 (42.9)	23 (35.9)
III/IV	48 (57.1)	41 (64.1)
B symptoms	49 (58.3)	39 (60.9)
Bulky disease (≥7 cm)	28 (33.3)	22 (34.4)
IPI risk groups		
Low/low–intermediate	52 (61.9)	37 (57.8)
Intermediate–high/high	32 (38.1)	27 (42.2)

Among the 84 patients with DLBCL, 20 patients received CHOP (6–8 cycles), and 64 patients received R-CHOP therapy (6–8 cycles). After R-CHOP therapy, radiotherapy was applied in 15 (23.4 %) patients with bulky or residual masses. Response to the therapy was assessed using the International Working Group criteria [29]. Patients were systematically followed up from diagnosis to September 2010. The overall characteristics of the 84 patients eligible for this study and the 64 R-CHOP-treated patients are shown in Table 1.

Informed consent was obtained from all DLBCL patients or patients' close relatives. The study was approved by the Ethics Committee of MMA.

Methods

Extraction of DNA

Each patient's DNA was extracted from whole blood by Blood Prep™ Chemistry for ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems, USA).

TNF-α −308 genotyping

TNF-α −308 genotypes were determined by restriction fragment length polymorphism (RFLP) of polymerase chain reaction (PCR) products [30]. PCR products were digested with restriction endonuclease *Nco*I according to the manufacturer's recommendations (Fermentas, Lithuania). Digested DNA was analyzed on 10 % polyacrylamide gels (PAGs) after electrophoresis and silver nitrate staining [31]. G at position 308 was represented with the presence

of two bands (325 and 20 bp), while A at 308 was visualized as a 345-bp band on the gel. All samples were analyzed in duplicate.

IL-10 −1082, IL-10 −819 and TGF-β Leu10Pro genotyping

The genotyping for the single-base-pair polymorphisms at these loci was performed by an amplification refractory mutation system (ARMS) PCR method previously described by Perrey and co-workers [32]. This method involves a specific sense primer complementary to the wild-type allele or a sense primer complementary to the variant allele in combination with the generic antisense primer. For each sample and each locus, two parallel PCR reactions were performed. As an internal control, primers amplifying a human growth hormone sequence were added to each PCR reaction. The presence/absence of PCR products was visualized on 2 % agarose gels after electrophoresis and ethidium bromide staining.

For each of these gene loci we chose ten samples (blind controls) and repeated the analysis.

IL-10 −3575 genotyping

IL-10 −3575 genotypes were determined by TaqMan® SNP genotyping assay rs1800890 (Applied Biosystems, USA) on a 7500 Real Time PCR System (Applied Biosystems, USA). All samples were analyzed in duplicate.

Statistical analysis

The association between SNP genotypes and clinical characteristics at diagnosis and outcome were analyzed using the chi-squared test. The prognostic relevance of different parameters was examined by stepwise logistic regression analysis. Event-free survival (EFS) was defined as the time from first day of treatment to progressive disease under therapy (PD), relapse or death from lymphoma. Overall survival (OS) was defined as the time from first day of treatment to death from any cause or to date of last follow-up. Survival curves were generated using the method of Kaplan and Meier and compared by the log-rank test. The Cox proportional hazards model was used to estimate effect of gene polymorphisms along with DLBCL characteristics for OS. The Cox model included only parameters that showed statistical significance in univariate analyses.

Statistical analyses were performed using SPSS software for Windows, version 15 (SPSS, Inc., Chicago, IL, USA). $p < 0.05$ was considered to indicate statistical significance.

Results

Genotype analysis and clinical characteristics

The observed genotype frequencies *IL-10* (*−3575*, *−1082*), *TNF-α* and *TGF-β* were in Hardy–Weinberg equilibrium.

Factors examined for prognostic significance included age (≤60years vs. >60 years), sex, Ann Arbor stage (I/II vs. III/IV), B symptoms, bulky disease, and IPI risk score (low/low–intermediate vs. intermediate–high/high). Table 2 summarizes the distribution of genotypes with respect to the clinical characteristics of disease.

IL-10 −1082 A/G and *TNF-α −308* G/A polymorphisms were not associated with established prognostic factors (shown in Table 2). We observed an association between *IL-10 −3575* and *TGF-β Leu10 Pro* polymorphism and clinical characteristics of disease. For *IL-10 −3575* polymorphism, patients presenting with B symptoms had TA/AA genotypes (70.7 %) more frequently than TT genotype (45.5 %) [odds ratio (OR) 2.89, 95 % confidence interval (CI) 1.11–7.57; $p = 0.03$]. Analyses of the distribution of *TGF-β* genotypes with respect to the stage and IPI score have shown significantly more frequent stage III/IV (OR 4.65, 95 % CI 1.33–16.19; $p = 0.016$) and intermediate–high/high IPI score (OR 5.37, 95 % CI 1.45–20.0; $p = 0.012$) in patients with the Pro allele variant (LeuPro/ProPro genotypes) than in carriers of the LeuLeu genotype.

Genotype analysis and outcome of disease

We analyzed the association of *IL-10*, *TNF-α* and *TGF-β* gene polymorphisms with the outcome (OS, EFS) in the cohort of 64 DLBCL patients treated with R-CHOP therapy.

In this group of patients, 46 (73 %) achieved complete remission (CR), 11 (17.5 %) experienced disease progression during therapy (PD) and 15 (23.4 %) patients died. With a median follow-up of 36 months (range, 0.5–77), the 3-year OS and EFS for all R-CHOP-treated patients were 71.7 % (95 % CI 63.7–79.7) and 59.0 % (95 % CI 49.5–68.5), respectively.

Analysis of genotypes and outcome did not show any association of *IL-10* (*−3575*, *−1082*) and *TGF-β Leu10-Pro* polymorphisms with outcome for patients with DLBCL (data not shown).

However, we observed an association between the *TNF-α –308* G/A polymorphism and outcome in the 64 R-CHOP treated patients. The carriers of the GG genotype showed higher sensitivity to R-CHOP therapy. Clinical characteristics of GG and GA/AA carriers and their response to therapy are shown in Table 3. Patients with GA/AA genotypes had significantly decreased OS compared to homozygous G-allele carriers (3-year OS 61.3 % for AG/

Table 2 Association between *IL-10* (*−3575*, *−1082*), *TNF-α −308* and *TGF-β* polymorphisms and clinical characteristics of DLBCL patients

Genotype	*N* (%)	Sex M/F (%)	*p*	Age >60 *N* (%)	*p*	CS III/IV *N* (%)	*p*	B symptoms *N* (%)	*p*	Bulky disease *N* (%)	*p*	IPI 3–5 *N* (%)	*p*
IL-10 −1082	83	42/41											
AA	23 (27.7)	56.5/43.5	0.60	8 (34.8)	0.91	10 (43.5)	0.13	13 (56.5)	0.88	7 (30.4)	0.80	8 (34.8)	0.76
AG + GG	60 (72.3)	48.3/51.7		19 (31.7)		37 (61.7)		35 (58.3)		20 (33.3)		23 (38.3)	
IL-10 −3575	74	36/38											
TT	33 (44.6)	42.4/57.6	0.89	9 (27.3)	0.39	19 (57.6)	0.76	15 (45.5)	0.03[a]	9 (27.3)	0.39	14 (42.4)	0.77
TA + AA	41 (55.4)	53.7/46.3		15 (36.6)		25 (61.0)		29 (70.7)		15 (36.6)		16 (39.0)	
TNF-α −308	83	42/41											
GG	43 (51.8)	53.5/46.5	0.58	17 (39.5)	0.16	27 (62.8)	0.34	27 (62.8)	0.47	17 (39.5)	0.25	19 (44.2)	0.27
GA + AA	40 (48.2)	47.5/52.5		10 (25.0)		21 (52.5)		22 (55.0)		11 (27.5)		13 (32.5)	
TGF-β c10	51	22/29											
LeuLeu	18 (35.3)	44.4/55.6	0.89	4 (22.2)	0.41	8 (44.4)	0.016[b]	10 (55.6)	0.31	6 (33.3)	0.83	4 (22.2)	0.012[c]
LeuPro/ProPro	33 (64.7)	42.2/57.6		13 (33.3)		26 (78.8)		23 (69.7)		12 (36.4)		20 (60.6)	

[a] B symptoms were more frequently present in patients with *IL-10 −3575* TA/AA than in patients with TT genotype ($p = 0.03$, *OR* 2.89, 95 % *CI* 1.11–7.57)

[b] The frequency of clinical stage III/IV was significantly higher in patients with *TGF-β* LeuPro/ProPro genotypes than in patients with LeuLeu genotype ($p = 0.016$, *OR* 4.65, 95 % *CI* 1.33–16.12)

[c] *TGF-β* LeuPro/ProPro carriers have an increased risk of IPI score ≥3 ($p = 0.012$, *OR* 5.37, 95 % *CI* 1.45–20.0) compared to LeuLeu carriers

Table 3 Clinical characteristics and response to R-CHOP therapy with respect to *TNF-α −308* genotypes

Characteristics	GG (34 patients) *N* (%)	GA + AA (30 patients) *N* (%)	*p*
Sex (M/F)	17/17	16/14	0.98
Age >60 years	10 (29.4)	9 (30.0)	0.96
CS III/IV	22 (64.7)	19 (63.3)	0.91
B symptoms	22 (64.7)	17 (56.7)	0.51
Bulky disease	15 (44.1)	7 (23.3)	0.08
IPI 3–5	15 (44.1)	12 (40.0)	0.73
Overall response	32 (94.1)	21 (70.0)	0.04
Complete response	26 (76.5)	20 (66.7)	0.35
PD under treatment	2 (5.9)	9 (30.0)	0.04

PD progressive disease

GG vs. 82.5 % for GG genotype; $p = 0.048$) (Fig. 1b). With regard to EFS, there was a trend toward a less favorable outcome for GA/AA genotypes (3-year EFS 48.1 %) than GG genotype (3 years 70 %), but statistical significance was not reached ($p = 0.07$) (Fig. 1a).

In multivariate analysis of prognostic factors for survival, including the clinical characteristics (IPI ≥3, B symptoms, male sex) and *TNF-α −308* G/A, GA/AA genotypes (OR 0.23; 95 % CI 0.07–0.78; $p = 0.018$) and advanced IPI score (OR 0.08; 95 % CI 0.02–0.43; $p = 0.003$) were significantly associated with the OS (Table 4).

We also analyzed the association between the polymorphism combinations and the survival of R-CHOP-treated patients. The results obtained were not statistically significant, probably due to the small group of patients.

Discussion

The present study examined the association of *IL-10* (*−3575*, *−1082*), *TNF-α −308* and *TGF-β Leu10Pro* polymorphisms with the clinical characteristics and outcome of DLBCL patients treated with R-CHOP therapy.

Published data indicate that common genetic variants in immune/inflammatory response genes are associated with the risk of developing lymphomas [21–24]. The largest multicentre epidemiological study identified *TNF-α −308* G/A and *IL-10 −3575* T/A as a risk polymorphism for development of DLBCL [20]. However, only a limited number of studies have analyzed the clinical characteristics and treatment outcome of DLBCL in association with *IL-10*, *TNF-α* and *TGF-β* polymorphisms [25–27, 33].

To date, only a few studies have been published that addressed the association of *TNF-308* polymorphism and

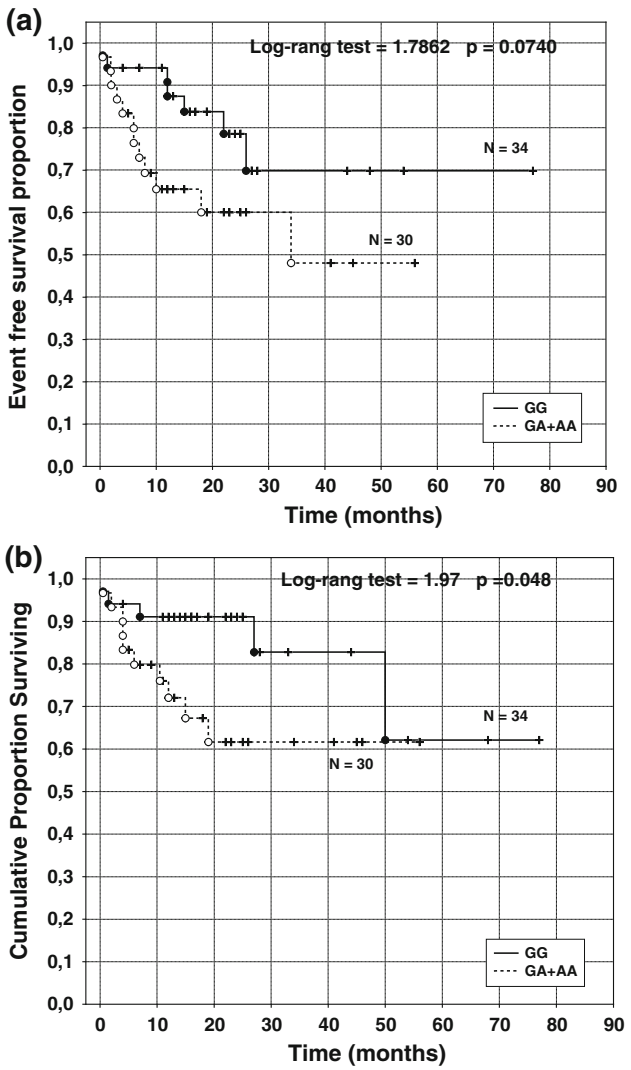

Fig. 1 Event-free survival (**a**) and overall survival (**b**) of DLBCL patients treated with R-CHOP in relation to *TNF-α −308* G/A polymorphism (GG vs. GA + AA)

Table 4 Multivariate analysis of prognostic factors for overall survival in DLBCL patients

Factor	Odds ratio (95 % CI)	*p*
Male sex	3.4 (0.8–13.2)	0.082
B symptoms	0.6 (0.12–3.3)	0.577
IPI ≥3	0.08 (0.02–0.43)	0.003
GA + AA versus GG	0.23 (0.07–0.78)	0.018

TNF-α −308 GA/AA genotypes (OR 0.23, $p = 0.018$) and intermediate–high/high IPI score (OR 0.08, $p = 0.003$) were significantly associated with OS

prognosis of patients with DLBCL [26, 33]. Warzocha and co-workers [26] reported that an extended haplotype in TNF and LT-α was associated with higher TNF production at the time of lymphoma diagnosis, and DLBCL patients with two or more TNF/LT-α high-producing alleles had lower progression-free survival and OS. In our study, we observed a relationship between *TNF-α −308* G/A polymorphism and outcome of 64 patients treated with R-CHOP. We found significantly worse survival in patients treated with R-CHOP who had AG/AA genotypes than carriers of the *TNF-α −308* GG genotype. The *TNF-α −308* A allele is associated with higher constitutional and inducible expression of TNF-α [9]. In-vitro studies have suggested that high levels of TNF-α can reduce cellular sensitivity to apoptosis-inducing chemotherapeutic agents and contribute to the emergence of drug-resistant disease [34]. However, it has been reported that rituximab inhibits the constitutive NF-κB signaling pathway in selected non-Hodgkin lymphoma B-cell lines, leading to increased sensitivity to chemotherapy [35]. Results of studies that have investigated the influence of *TNF-α* and *LT-α* gene polymorphisms on infections and predisposition to death from sepsis suggested that patients with high-producing genotypes can manifest significantly more severe inflammatory reactions [36]. However, in our patients R-CHOP therapy was not accompanied by pronounced toxic effects or life-threatening infections or required a significant delay in immunochemotherapy. In the present study, carriers of *TNF-α* GG showed higher sensitivity to R-CHOP therapy than carriers of the AG/AA genotypes. In addition, patients with AG/AA genotypes showed significantly more resistant/PD during the early course of therapy and died from lymphoma progression within the first 18 months.

Furthermore, we observed a positive association between *IL-10 −3575* TA/AA genotypes and the presence of B symptoms in DLBCL patients. Several reports have demonstrated that increased serum levels of IL-10 were associated with poor outcome and adverse prognostic factors in patients with aggressive non-Hodgkin lymphoma [25, 37]. However, previous in-vitro studies have suggested that the *IL-10 −3575* A allele is associated with low, and the T allele with high production of IL-10 [38]. There is only one study on DLBCL, published by Kube et al. [27], reporting that the *IL-10 −3575* AA carriers have an increased risk of displaying intermediate–high/high IPI score. In addition, we found no difference in OS or EFS in relation to *IL-10 −3575* T/A and *IL-10 −1082* A/G polymorphism. Similar findings were also reported by other investigators [27, 39, 40].

Several polymorphisms in the *TGF-β* gene have been reported [41]. Dunning and co-workers [42] found that TGF-β Pro variant of codon 10 was associated with a higher level of serum TGF-β and increased risk of invasive breast cancer. This and other studies have suggested the importance of the *TGF-β Leu10Pro* polymorphism for the incidence and clinical course of various diseases [43–45]. To date, only a few studies have analyzed the clinical

course and outcome in patients with non-Hodgkin lymphoma in association with *TGF-β* polymorphisms. In the present study, we found that the Pro variant (LeuPro/Pro-Pro genotypes) of TGF-β codon 10 was associated with the unfavorable phenotypic features of DLBCL—advanced clinical stage III/IV and intermediate–high/high IPI score. The relationship between *TGF-β Leu10Pro* and more aggressive non-Hodgkin lymphoma had been recognized previously by Mazur and co-workers [46]. However, in both studies, the present one and Mazur et al., *TGF-β* polymorphism was not a prognostic parameter that affected survival of lymphoma patients.

In summary, the present study included DLBCL patients who were diagnosed, uniformly treated, and controlled in one institution. In this ethnically matched group of patients, the frequencies of the analyzed genotypes were similar to those previously reported for other European populations. Our results indicate an association between *IL-10 −3575* and *TGF-β Leu10Pro* gene polymorphisms and clinical characteristics. Furthermore, *TNF-α −308* was associated with treatment outcome of DLBCL patients treated with R-CHOP. According to our knowledge, there are no published studies on the association of cytokine gene polymorphisms and survival of DLBCL patients treated with R-CHOP. Herein, we report that the *TNF-α −308* A allele is associated with adverse response to R-CHOP therapy. In addition to the *TNF-α −308* A allele, unfavorable IPI score (intermediate–high/high) remained an independent risk factor for survival of the patients analyzed. This finding has possible clinical implications regarding treatment. In a selected group of patients, the use of immunomodulatory agents or TNF inhibitors may be an attractive approach [47]. Preclinical and early clinical data demonstrated a synergistic action of immunomodulatory drugs (especially lenalidomide) and rituximab [48–50]. Considering these findings, immunomodulatory therapy, alone or in combination with rituximab, represents a promising strategy for improving prognosis in DLBCL.

Conflict of interest The authors declare that they have no conflict of interest. All financial and material support for this research and work was obtained from Military Medical Academy, Belgrade, Serbia.

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