

Fc-Gamma-Receptor IIIa Polymorphism and Gene Expression Profile Do Not Predict the Prognosis in Diffuse Large B-cell Lymphoma Treated with R-CHOP Protocol

László Váróczy · Erika Zilahi · Ágnes Gyetvai ·
Béla Kajtár · Lajos Gergely · Sándor Sipka · Árpád Illés

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Abstract The addition of rituximab to conventional chemotherapy has significantly improved the treatment outcome in diffuse large B-cell lymphoma. However, differences in treatment response and survival data can be observed independently from the International Prognostic Index scores. The current study evaluated the impact of Fc-gamma-receptor IIIa polymorphism and gene expression profile on the response of DLBCL patients to R-CHOP therapy as well as on their survival results. Fifty-one patients were involved, thirty-two females, nineteen males, their median age was 53.1 years. The FCGR3A polymorphism at the 158. amino acid position determined with PCR method showed the following results: VV: 12 cases (23.5%), VF: 29 cases (56.8%) and FF: 10 cases (19.6%), respectively. There was no significant difference between the treatment responses of the three groups. The event-free survival data were less favorable in the F-allele carriers than in V/V homozygous patients, but without any significance, and the overall survival

curves were almost the same. As for the gene expression profile, 20 patients' biopsy specimens showed germinal center and 31 showed non-germinal center characteristics. Treatment results did not differ from each other in the two groups. Both the event-free and the overall survival data were more favorable in the GC group, however the differences were not significant. Our results contest the predictive value of Fc-gamma-receptor IIIa polymorphism and gene expression profile in diffuse large B-cell lymphoma.

Keywords Diffuse large B-cell lymphoma · Rituximab · Fc-gamma-receptor IIIa polymorphism · Gene expression profile · Treatment response · Survival

Abbreviations

ABC	Activated B-cell
ADCC	Antibody dependent cellular cytotoxicity
bp	Base pair
CD	Cluster designation
DLBCL	Diffuse large B-cell lymphoma
DNA	Desoxy ribonucleic acid
EFS	Event-free survival
F	Phenylalanine
FCGR3A	Fc-gamma-receptor IIIa
GC	Germinal center
IPI	International prognostic index
mRNA	Messenger ribonucleic acid
NK	Natural killer
OS	Overall survival
PCR	Polymerase chain reaction
R-CHOP	Rituximab, cyclophosphamide, H-daunorubicine, oncovin, prednisolone
V	Valine

L. Váróczy (✉) · L. Gergely · Á. Illés
3rd Department of Medicine, Institute for Medicine, Medical and
Health Science Center, University of Debrecen,
Móricz Zs. 22.,
4032 Debrecen, Hungary
e-mail: laszlo.varoczy@gmail.com

E. Zilahi · Á. Gyetvai · S. Sipka
Regional Laboratory for Immunology, Medical and Health
Science Center, University of Debrecen,
Móricz Zs. 22.,
4032 Debrecen, Hungary

B. Kajtár
Department of Pathology, University of Pécs,
Pécs, Hungary

Introduction

Therapeutic response and survival data of patients with diffuse large B-cell lymphoma (DLBCL) have improved significantly in the recent years, thanks to the novel diagnostic and treatment methods. R-CHOP protocol (rituximab 375 mg/m² on day 0, cyclophosphamide 750 mg/m² on day 1, vincristine 2 mg on day 1, doxorubicine 50 mg/m² on day 1 and prednisolone 60 mg/m² on days 1–5) is commonly used as a first-line regimen in the treatment of DLBCL. The ratio of therapeutic response is over 80% to this protocol [1, 2]. However, there are some patients who do not respond well or even progress on this therapy. The International Prognostic Index (IPI) is still widely used to determine the patients' prognosis when starting the treatment, but therapeutic responses cannot be predicted only by IPI in all cases [3]. There might be some other general or individual risk factors that strongly influence the prognosis of DLBCL patients. These can include special genetical alterations that may affect the whole body or only the tumor cells [4].

Antibody dependent cellular cytotoxicity (ADCC) plays a key role in the B-cell killing effect of rituximab. The main effector cells of ADCC include natural killer (NK) cells and macrophages. These cells bind to the antibodies with the help of their Fc receptors on their surface, which results in their activation and the release of toxic products [5, 6]. It has been detected that the affinity of the effector cells to the monoclonal antibody (rituximab) is genetically determined. A nucleotide substitution at position 559 of Fc-gamma-receptor III a (FCGR3A) predicts either a valine (V) or a phenylalanine (F) at amino-acid position 158. In vitro studies suggest that the affinity of NK-cells to rituximab is much higher in those individuals who have a V/V genotype than in V/F heterozygous or F/F homozygous patients. The relative prevalence and the affinity to the anti-CD20 antibody of the three genotypes are: V/V 20% and 100%, V/F: 45% and 67%, F/F 35% and 51%, respectively [7, 8].

Gene expression profile of lymphoma cells can be another important factor that influences the prognosis of patients with DLBCL. Recently, the Lymphoma/Leukemia Molecular Profiling Project has analyzed the global mRNA profile of malignant lymphomas, using spotted- or oligonucleotide arrays. It has become clear that DLBCL with a mRNA signature resembling germinal center B-cells (GCB DLBCL) show a more indolent behavior than in DLBCL in which the mRNA signature more closely resembles that seen in vitro activated B-lymphocytes (ABC or non-GC) DLBCL [9, 10]. Hans et al. proposed an algorithm based upon the expression of CD10, Bcl-6 and MUM-1 to discriminate between GCB and non-GCB DLBCL (Fig. 1) [11].

In this study, our aim was to investigate if Fc-gamma-receptor IIIa polymorphism and gene expression profile

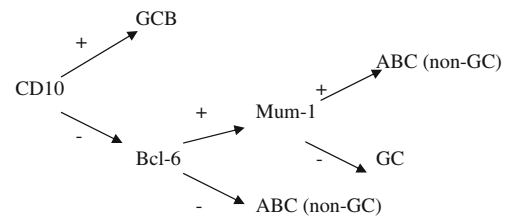


Fig. 1 Decision algorithm as described by Hans et al. [11]

have an impact on the therapeutic response and survival data in our DLBCL patients.

Patients and Methods

Clinical Data

The clinical files of DLBCL patients were reviewed with particular reference to age, sex, IPI, response to treatment and survival. Examining the survival rates, overall survival (OS) was determined by consideration of death events due to any reasons, while event-free survival (EFS) was determined by consideration of death events, relapses or disease progression that indicated further treatment. Descriptive statistical analysis was used to characterize the patient populations. Normality of the parameters were examined applying the Wilk-Saphiro test. Comparing two groups, F probe and *t* test were administered by normal distribution of the parameters, otherwise the non-parametrical Mann–Whitney test was applied. Differences were significant if probability level was less than 5% ($p < 0.05$). Survival rates were calculated using the Kaplan-Meier's method, while the survival data were compared using the log-rank test.

Determination of Fc-Gamma-Receptor IIIa Polymorphism

Genomic DNA Extraction

High molecular weight DNA for genotyping was extracted from peripheral blood samples according to the manufacturer's recommendation using a QiaAmp DNA blood mini kit (Qiagen GmbH, Germany).

Genotyping by Real-Time PCR

Genotyping was employed using a real-time PCR method that proves to be faster than the conventional methods based on restriction enzyme digestion. Genotyping of the single nucleotide substitution ($G \rightarrow T$, *rs396991*) was carried out using the allelic discrimination assay. PCR primers and TaqMan probe specific for FCGR3A-158 polymorphism were purchased from Applied Biosystems (Foster City, CA). The assay enables scoring of both alleles

in a single well. Real-time PCR was performed in a Corbett Rotor-Gene RG-3000 equipment.

The PCR reaction was carried out in a 20 µl reaction volume containing TaqMan Universal Master Mix (2X, 4331182, Applied Biosystems), TaqMan genotyping Assay (20X) and optimized quantities of genomic DNA. The Universal Master Mix contained AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP, passive reference, and optimized buffer components. Reactions were set up in duplicate. Thermal cycling was initiated by incubation at 95°C for 10 mins for optimal AmpErase UNG activity and activation of AmpliTaq Gold DNA polymerase. After this initial step, 40 cycles of PCR were performed. Each PCR cycle consisted of heating to 92°C for 15 s for melting, and to 60°C for 1 min for annealing and extension.

PCR-Based Allele-Specific Restriction Assay

Nested PCR was performed as previously described by Dall'Ozzo et al. (2003) with slight modifications. Two FCGR3A-specific primers (5' – ATATTTACAGAATGGCA CAGG – 3' and 5' – GACTTGGTACCCAGGTTGAA – 3') were used to amplify a 1.2 kb fragment containing the polymorphic site. The second PCR used primers (5' – ATCAGATTCGATCCTACTTCTGCAGGGGGGCAT – 3' and 5' ACGTGCTGAGCTTGAGTGATGGTGATGTTTAC – 3') amplifying a 94 bp fragment. The amplified DNA was then digested with 10 U NlaIII (Fermentas) at 37°C for 1 h and separated by electrophoresis on 3% agarose gel. PCR products were visualised under UV transillumination. Only one undigested band (94 bp) was visible for homozygous FCGR3A-158 F individuals, three bands (94, 61 and 33 bp) were seen in heterozygous individuals, but two digested bands (61 and 33 bp) were obtained for homozygous FCGR3A-158 V individuals.

Immunohistochemistry

Sections of formalin fixed paraffin embedded biopsies sampled from the affected DLBCL patients were examined

by immunohistochemistry for CD10 (clone: 56 C6; Novocastra, Newcastle Upon Tyne, UK), bcl-6 (clone: PG-B6p; DAKO, Glostrup, Denmark), MUM1 (MUM1p; DAKO) and Bcl-2 (124; DAKO). The specimens were scored positive for CD10, Bcl-6 and MUM1 if a minimum of 30% of the neoplastic cells were labeled, whereas the cutoff levels for Bcl-2 was 50%.

Results

Patient Characteristics

Fifty-one patients with diffuse large B-cell lymphoma were involved in the study. Thirty-two of them (62.7%) were females, nineteen of them (37.3%) were males, the median age was 53.1 years (18–86) at the time of diagnosis. They were all treated with R-CHOP chemotherapy between 1st January 2007 and 30th June 2010. They received 1–8 cycles, the therapy was administered biweekly in 32 cases (R-CHOP-14 protocol) and every 3 weeks in 19 cases (R-CHOP-21 protocol). The prevalence of the three FCGR3A genotypes were: VV: 12 cases (23.5%), VF: 29 cases (56.8%) and FF: 10 cases (19.6%), respectively. As for the gene expression profile, 20 biopsy specimens showed germinal center and 31 showed non-germinal center characteristics.

Treatment Response

Treatment response data are summarized in Table 1 (FCGR3A polymorphism) and Table 2 (gene expression profile). Clinical features did not differ significantly in each patient group. Moreover, there was no significant difference between therapeutic response data including overall response rate (complete and partial response) and stable/progressive disease.

Survival Data

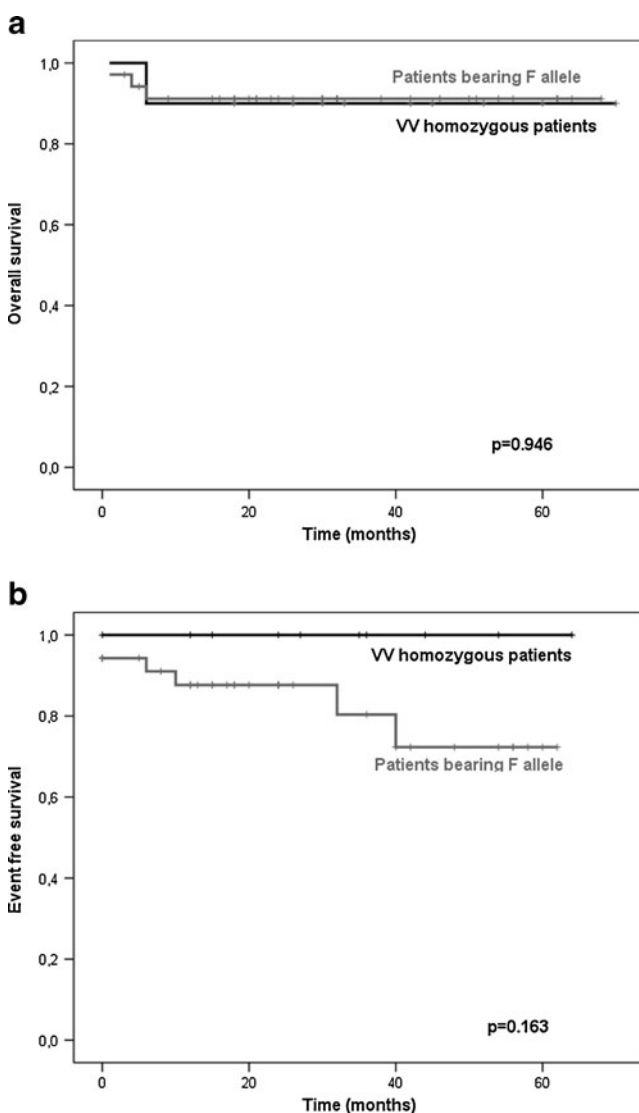
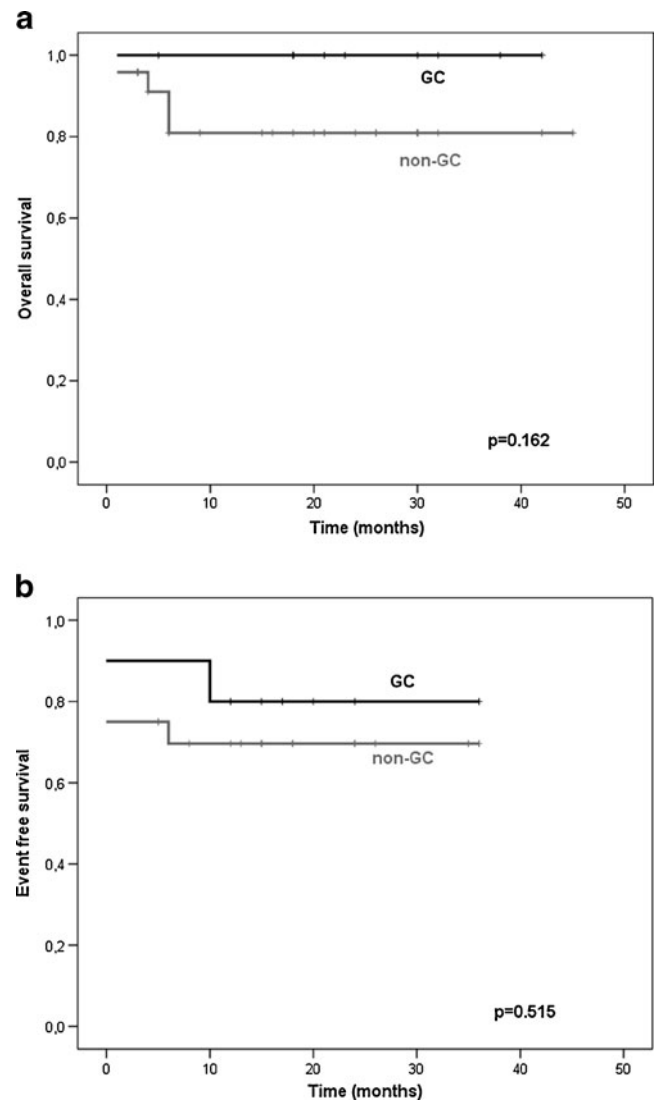
Survival data are shown in Figs. 2a, b and 3a, b. The median follow-up of the patients was 2.4 years. The overall

Table 1 Patient characteristics and treatment outcomes according to the FCGR3A genotypes

	FF	VV	VF	<i>p</i>
Number of patients (%)	10 (19.6)	29 (56.8)	12 (23.5)	-
Sex, no. female/males	7/3	16/13	7/5	NS
Median age (range)	62.5 (37–70)	49.82 (19–83)	53.5 (25–86)	NS
Mean IPI score	1.8	2.1	1.7	NS
Response no. (%)				
CR	7 (70)	20 (69)	10 (83)	NS
PR	1 (10)	5 (17)	0 (0)	NS
SD/PD	2 (20)	4 (14)	2 (17)	NS
OR (PR+CR)	8 (80)	25 (86)	10 (83)	NS

Table 2 Patient characteristics and treatment outcomes according to the gene expression profiles

	GC	Non-GC	<i>P</i>
Number of patients (%)	20 (39)	31 (61)	-
Sex, no. female/males	14/6	16/15	NS
Median age (range)	51.6 (19–75)	54.5 (18–86)	NS
Mean IPI score	1.8	2.06	NS
Response no. (%)			
CR	15 (75)	21 (68)	NS
PR	3 (15)	4 (13)	NS
SD/PD	2 (10)	6 (19)	NS
OR (PR+CR)	18 (90)	25 (81)	NS

**Fig. 2** **a** Overall survival curves according to the FCGR3A polymorphism **b** Event-free survival curves according to the FCGR3A polymorphism**Fig. 3** **a** Overall survival curves according to the gene expression profile **b** Event-free survival curves according to the gene expression profile

survival curves of 158-V homozygous patients and F-allele carriers were very similar. As for the event free survival data, the results were more favorable in the V/V group, however, the difference was not significant. Considering the gene expression profile, there were no significant differences between the OS and EFS data in the GC and non-GC groups.

Discussion

The addition of rituximab to conventional chemotherapy has significantly improved the treatment outcome of several B-cell lymphoma subtypes. Although the exact mechanism of action of rituximab is not fully known, it likely includes ADCC, complement-dependent cytotoxicity and induction

of apoptosis. Focusing on ADCC, the binding of rituximab to CD20-positive tumor cells allows the recruitment of effector cells such as NK cells and macrophages [12]. These effector cells express Fc receptors that bind them to the antibody-covered B-cells. Former *in vitro* studies revealed that the level of ADCC activity depends on the genetical polymorphism of Fc-gamma-receptor IIIa [7, 8]. However, the role of FCGR3A polymorphism is controversial in treatment outcome. In 2002 Cartron et al. examined the magnitude of FCGR3A polymorphism in follicular lymphoma patients treated with single agent rituximab and reported that objective therapeutic responses were significantly better in FCGR3A-158 V homozygous individuals than in FCGR3A-158 F carriers [13]. Similar results were published by Treon et al. who examined the efficacy of rituximab therapy in patients with Waldenström's macroglobulinaemia [14]. Contrary, FCGR3A polymorphism did not predict response to rituximab in B-cell chronic lymphocytic leukaemia [15]. When examining the overall response rate and survival data in follicular lymphoma patients treated with R-CHOP immuno-chemotherapy, there was no correlation found with FCGR3A polymorphism [16]. Meanwhile, Kim et al. showed that FCGR3A-158 V homozygous patients with diffuse large B-cell lymphoma achieved a significantly higher complete response rate to R-CHOP treatment than FCGR3A-158 F carriers [17].

The first data about the importance of immunohistochemistry profiling in DLBCL were published in the early 2000s when CHOP chemotherapy alone was administered in this disease. These studies indicated that the expression of GCB related markers (CD10, Bcl-6) at the mRNA level is a favorable parameter, in contrast to Bcl-2, which is an adverse prognostic factor [18]. However, treatment outcome results changed when the addition of rituximab to conventional chemotherapy became a routine practice. Recent investigations have indicated that differences in survival between GCB and non-GCB DLBCL are eliminated when rituximab is added to CHOP treatment [19, 20]. Furthermore, it seems that rituximab also significantly improves survival of both Bcl-6-negative and Bcl-2-positive diseases, which means that it is especially useful in high-risk DLBCL [21].

Our results showed that determining either the Fc-gamma-receptor polymorphism or the gene expression profile could not predict the prognosis of our DLBCL patients. The overall response rate was at least 80% both in the homozygous V/V genotype group and among the F-allele carriers. The therapeutic response was a bit more favorable in patients having GC-type disease than in those with non-GC DLBCL (overall response rates: 90% vs 81%), however the difference was not significant. It is important to emphasize that our patients were relatively young, their median age (53.1 years) was lower than it was reported from other centers. Thirty-two patients (62.7%)

received R-CHOP therapy biweekly (R-CHOP-14 protocol) as this intensified therapy was expected to provide the most favorable treatment outcomes. Moreover, this therapeutical strategy may have contributed to the fact that patients having less favorable Fc-gamma-receptor genotype or gene expression profile have fallen into line with those having good prognostic factors in the point of treatment response data. We also analyzed the survival data of our DLBCL patients, however, the mean follow-up time was relatively short. No significant differences could be observed between the OS and EFS results, respecting either the FCGR3A polymorphism or the gene expression profile. These latter data are in concordance with the observations of other centers [16, 17].

In conclusion, the present study suggests that FCGR3A polymorphism and gene expression profile do not have an impact on the response to R-CHOP therapy in DLBCL patients.

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