



Immunological microenvironment gene expression in patients with diffuse large B cell non Hodgkin lymphoma

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ABSTRACT

Background: Non Hodgkin lymphoma (NHL) is one of the immune system cancers. The occurrence and progression of malignant lymphomas depends on cellular pathways deregulation. Understanding the relationship between the immune system at the genetic level and malignant transformation is critical to reach its etiology. **Objective:** The aim of this work is to evaluate the expression of five immune related genes (PD-1, FOXP3, GrA, GrB and CD11c) in patients with diffuse large B cell non Hodgkin lymphoma (DLBCL).

Materials and methods: This study was conducted on fifty patients with DLBCL and fifty sex and age matched apparently healthy subjects. The participants were subjected to these laboratory investigations: complete blood count, serum lactate dehydrogenase and β 2microglobulin (β_2M) levels and determination of PD-1, FOXP3, GrA, GrB and CD11c gene expressions.

Results: The results of this study revealed that PD-1, FOXP3, GrA, GrB and CD11c gene expressions were significantly increased in DLBCL patients.

Conclusion: Patients with DLBCL have variable PD-1, FOXP3, GrA, GrB and CD11c gene expressions levels, which are correlated with the overall survival (OS) indicating that they can be good predictors of outcome in these patients.

1. Introduction

Non-Hodgkin's Lymphoma (NHL) is a tumor that originates from B, T or Natural killer T (NKT) cells, and occurs via the malignant transformation of lymphocyte [1]. The mechanism depends on defect in apoptosis, proliferation, or differentiation in cellular pathways [2]. B-cell lymphomas represent most of non-Hodgkin lymphomas (about 85%) while T-cell lymphomas account for less than 15% [3]. Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of B-cell lymphomas with incidence of 42.5% [4].

Non-Hodgkin lymphoma (NHL) accounts for approximately 90% of cases of newly diagnosed lymphoma in the United States. About 95% of estimated deaths yearly because of lymphoma are due to NHL [5].

Immune function disorders as immunodeficiency and autoimmune diseases are associated with increased risk for malignant transformation [6]. A population of B cells which detect in autoimmune mice expresses integrin α_x chain (CD11c). Decrease the number of this population in vivo resulted in a low level of autoreactive antibodies. Thus CD11c has

an important role in autoimmune diseases, increasing the risk of lymphoma development [7].

An initial step in the malignant transformation is recurrent translocations, which occur during different steps of B-cell differentiation. Interestingly, these translocations alone are usually insufficient for development of cancer. As, genetic alterations of genes that control cell differentiation, proliferation, and survival are also required [8].

Antigen presenting cells (APCs) recognize cancer-specific antigens then activate T-cell responses against these specific antigens, the activated cytotoxic T cells kill cancer cells by the releasing cytotoxic agents such as perforin and granzyme (Gr). Killed cancer cells further release cancer-specific antigens. These sequential events are called cancer immunity cycle [9,10].

In cancer, Programmed cell death PD-1 and its ligand PD-L1 called the PD-1/PD-L1 system inhibits proliferation of T lymphocytes, release of cytokines, and cytotoxicity, which leads to exhaustion and apoptosis of tumor-specific T cells. PD-L1 is frequently overexpressed on the surface of different tumor types, thereby providing cancer cells the

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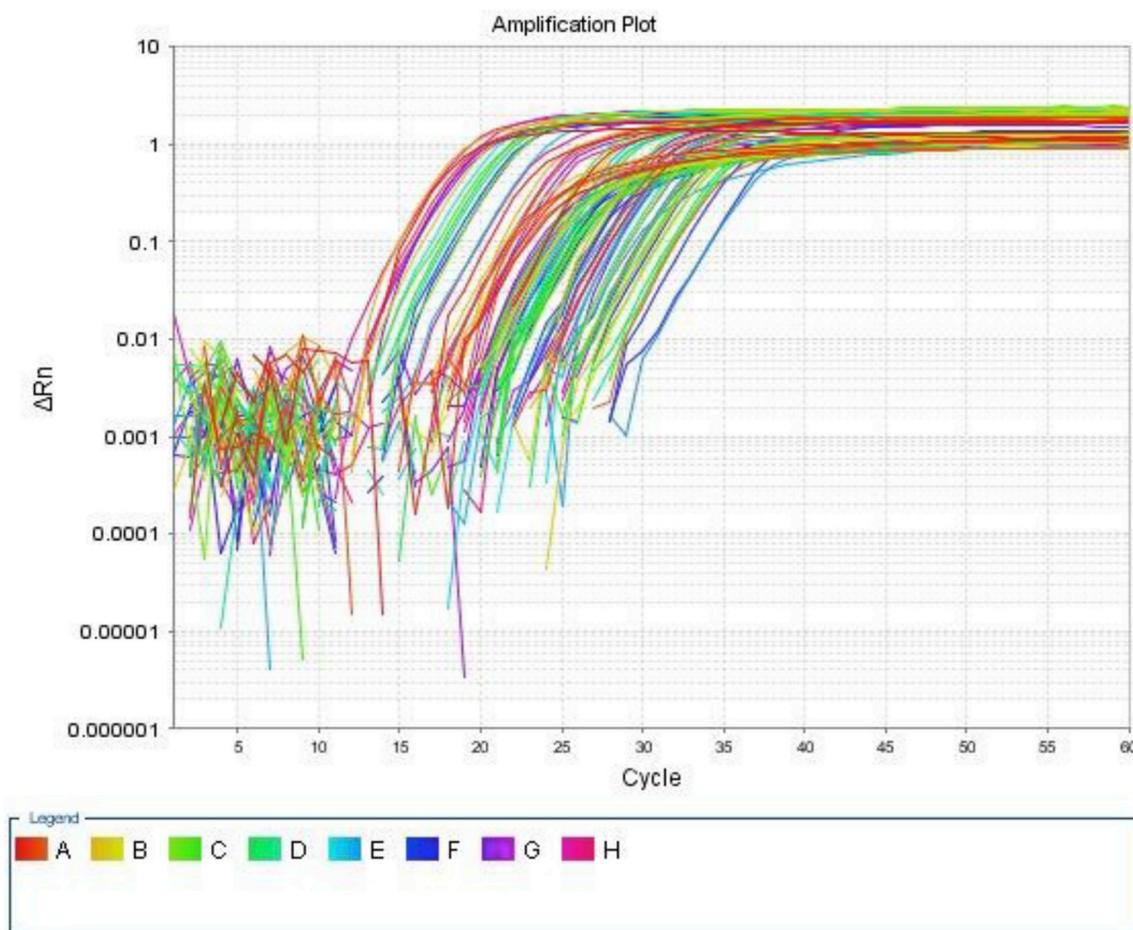


Fig. 1a. Amplification Plot of PD-1, GrA, FOXP3, GrB and CD11c gene expression.

opportunity to avoid immune response [11–13]. The function of T regulatory cells (Tregs) is regulated by a transcription factor called a Fork head boxP3 (FOXP3). Change of FOXP3 expression may leads to defect in Tregs developments [14].

This study aims to assess the expression of five immune related genes (PD-1, FOXP3, GrA, GrB and CD11c) in patients with diffuse large B cell non Hodgkin lymphoma.

2. Materials and methods

This work was achieved by cooperation between Biochemistry department, Faculty of Science, Menoufia University and Medical Biochemistry and Molecular Biology, department, Faculty of Medicine, Menoufia University. The subjects were selected from Oncology Department, Menoufia University Hospital and followed up in the period from February 2017 to June 2019. A written consent form approved by the Committee of Human Rights in Research in Menoufia University was obtained from every subject.

The study included 100 subjects categorized into two groups: **Group I (patients group)**: Included 50 subjects with DLBCL. Their mean age was 53.22 ± 15.30 years. They were subdivided into two subgroups: Group Ia: with tumor stage I and II. Group Ib: with tumor stage III and IV. **Group II (control group)**: Included 50 age and gender matched apparently healthy subjects. Their mean age was 52.24 ± 16.81 years. Patients, who have previously received chemotherapy for any cause, either recurrence or other primary tumours were excluded from the study.

Lymphoma was diagnosed by tissue biopsy either excisional lymph

node, image guided tissue biopsy, upper gastrointestinal endoscopic biopsy or bone marrow biopsy. The stages are described by Roman numerals I to IV according to Lugano classification [15]. While performance status as divided into five grades according to Cooperative Oncology Group (ECOG) [16]. The International prognostic index (IPI) remains the most important prognostic factor in NHL. It depends on five factors: age > 60 years, elevated serum lactate dehydrogenase (LDH), ECOG performance status ≥ 2 , Lugano stage III or IV, and number of involved extranodal sites > 1 [17].

- Complete history taking and general clinical examination. Clinical staging, pathological diagnosis, CT (neck, chest, abdomen and pelvis) with contrast and calculation of International prognostic index (IPI) were done for patients.

2.1. Methods

Five ml of venous blood were withdrawn from every subject and divided as such: 2 ml of blood were taken in a tube containing EDTA for complete blood count (CBC) and RNA extraction, while the other 3 ml were put in plain tube and allowed to clot for 30 min at room temperature then subjected to centrifugation for 10 min at 5000 rotation per minute (rpm) and the serum obtained was stored at -80°C until the time of assay.

- Complete blood count (CBC) was done by automated homogram. This included hemoglobin estimation (HB), red cell count (RBCs), white blood count (WBCs) and platelet count (coulter counter model

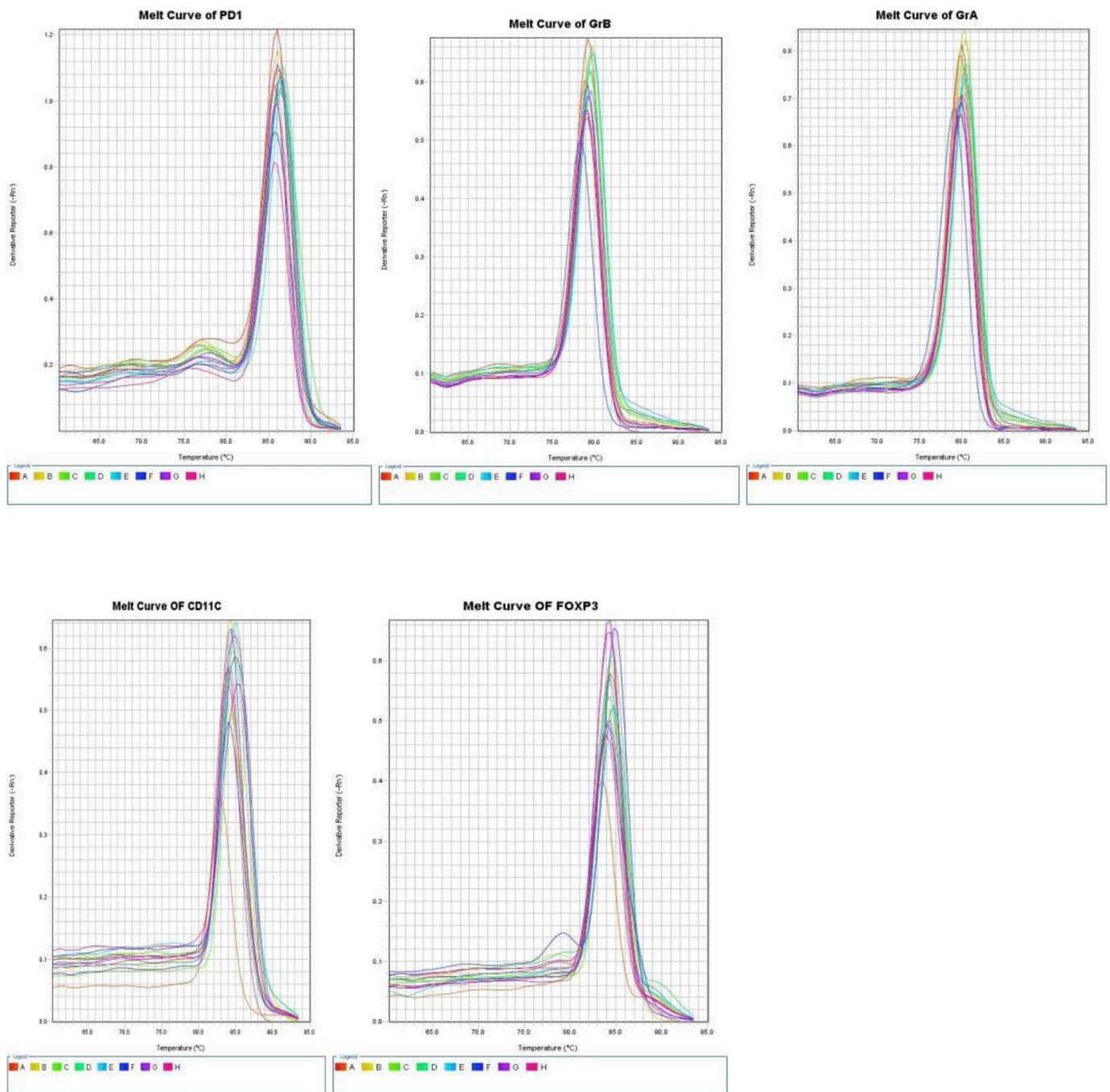


Fig. 1b. Melting curve of PD-1, GrA, FOXP3, GrB and CD11c gene expression.

Beckman 750, Int, U.S.A.).

- Serum lactate dehydrogenase (LDH) levels were determined photometrically by measuring the rate of NADH consumption which is directly proportional to the LDH activity in the sample (SPINREACT, SANT ESTEVE DE BAS (GI) SPAIN).
- Serum β 2 microglobulin was determined by enzyme linked immunosorbent assay method (ELISA), using EIAab[®] Human β 2 microglobulin ELISA kit, China.
- RNA extraction, cDNA synthesis, Real-Time PCR:

For evaluating the expression of PD-1,FOXP3, GrA, GrB and CD11c genes, the whole blood samples were subjected to RNA extraction using

Qiazol (Qiagen, Hilden, Germany) based on the manufacturer's instructions. The quality and quantity of extracted RNA was measured using Nanodrop spectrophotometer (NanodropTechnologies). The cDNA was synthesized from 10 μ g of RNA using MultiScribeFirst Strand cDNA Synthesis Kit (High capacity Reverse Transcription Kit, Thermofisher Scientific, Applied Biosystems, USA) according to the manufacturer's protocol. The synthesized cDNA was applied as a template for Real-Time PCR using SYBR Green II with low ROX (QuantiTect SYBR Green PCR Kit, Applied Biosystems, USA) in an Applied Biosystems 7500, software version 2.0.1.(Applied Biosystems, USA). The running program was as: 1 cycle at 95 °C for 5 min following 40 cycles at 95 °C for 5 S, 55 °C for 20 S and 60 °C for 35 S. The beta-actin

Table 1
Comparison between DLBCLgroup and control group regarding demographic data.

Age (Years) Mean ± SD	Patients (No = 50)		control (No = 50)		Test of sig. t- test 0.30	P-value 0.761
	N	%	N	%		
Gender						
Male	27	54.0	28	56.0	χ ² 0.42	0.841
Female	23	46.0	22	44.0		
Occupation					FE 2.14	0.967
Worker	4	8.0	5	10.0		
Farmer	11	22.0	11	22.0		
Teacher	7	14.0	4	8.0		
Housewife	11	22.0	8	16.0		
Driver	7	14.0	9	18.0		
Painter	3	6.0	4	8.0		
Engineer	3	6.0	4	8.0		
Doctor	2	4.0	3	6.0		
Nurse	2	4.0	2	4.0		
Smoking						
Yes	28	56.0	27	54.0		
No	22	44.0	23	46.0		

t = student t-test χ² = Chi-square test FE= Fisher's exact test.

Table 2
Clinical and pathological characteristics of patients group.

Variable	No %
Performance status	
0	19 (38.0)
1	23 (46.0)
2	8 (16.0)
Extranodalsite	
No	18 (36.0)
Yes	32 (64.0)
B symptoms	
No	32 (64.0)
Yes	18 (36.0)
Fate status	
dead	12 (24.0)
Live	38 (76.0)
Stage	
I	18 (36.0)
II	6 (12.0)
III	9 (18.0)
IV	17 (34.0)
B2 microglobulin level	
< 2 mg	0 (0.0)
< 4 mg	44 (88.0)
> 4 mg	6 (12.0)
IPI	
0	15 (30.0)
1	13 (26.0)
2	20 (40.0)
3	2 (0.040)

IPI = international prognostic index.

expression level was used as a house keeping gene to normalize the PD-1, FOXP3, GrA, GrB and CD11c gene expression levels and the comparative CT (2^{-ΔΔCt}) method was applied for analysis of gene expression. Fig. 1a and b shows the amplification plots and the melting curves. The designed primers which were used are listed below:

Gene name	Forward primer	Reverse primer
PD-1	5'- CGTGGCCTATCCACTCCTCA-3'	5'- ATCCCTTGTCCAGCCACTC-3'
FOXP3	5'-ACAACATGCGACCCCTTTACACC-3'	5'-GGTTGTGGCGGATGGCGTTCTTC-3'
GrA	5'- TTTCTGGCATCCTCTCTCA-3'	5'- GGGTCATAGCATGGATAGGG-3'
GrB	5'- TGGGGGACCCAGAGATTAATA-3'	5'- TTTCTGCCATAGAGACAATGC-3'
CD11c	5'- GGCCATGCACAGATACCAAGT-3'	5'- CTGGGGGTGCGATTTC-3'
B-Actin	5'-CCACTCTCCACCTTTGAC-3'	5'-ACCCTGTGCTGTAGCCA-3'

Table 3
Comparison of relative quantitative (RQ) gene expressions in patients and control groups.

	Patients (N = 50) Mean ± SD Median	Controls (N = 50) Mean ± SD Median	U test	P value
RQ of PD-1	9.66 ± 10.73 2.38	1.46 ± 1.57 1.00	1.82	0.044*
RQ of GrA	10.96 ± 13.54 3.90	3.35 ± 4.01 1.56	2.24	0.025*
RQ of GrB	13.48 ± 13.19 6.99	2.87 ± 4.82 0.65	5.75	< 0.001*
RQ of CD11c	7.90 ± 7.53 3.90	1.70 ± 1.43 1.08	5.46	< 0.001*
RQ of Foxp3	9.00 ± 8.43 4.76	1.82 ± 2.11 1.00	4.63	< 0.001*

SD = stander deviation * = significant U = Mann Whitney test.

2.2. Statistical analysis

IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp) was used for data analysis. Quantitative data were expressed as mean & standard deviation (X ± SD). P-value ≤ 0.05 was considered to be significant.

3. Results

There was a non-significant difference between patients with DLBCL and control group regarding demographic data including age, gender, occupation and smoking habit (Table 1).

Performance status of 23 patients (46.0%) was 1 while of 8 patients (16.0%) was 2. In 32 (64.0%) of patients, Extra-nodal site was present, while B symptoms was present in 18 patients (36.0%). The death is the fate in 12 patients (24.0%) at the end of the study (24 months). 48% of patients had tumor stage I and II while 52% had stage III and IV. β₂microglobulin level was < 4 mg in 88% of patients and > 4 mg in

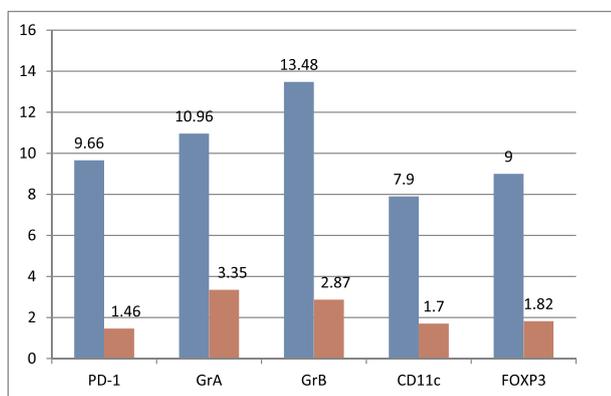


Fig. 2a. comparison of gene expression levels between patients and control groups.

Table 4
Comparison of relative quantitative (RQ) gene expressions in two patients' subgroups.

	Group Ia I&II (n = 24) Mean ± SD Median	Group Ib III&IV (n = 26) Mean ± SD Median	U test	P value
PD-1	0.99 ± 2.99 0.25	17.66 ± 8.87 16.94	5.28	< 0.001*
GrA	20.71 ± 13.74 19.60	1.97 ± 3.16 0.67	5.30	< 0.001*
GrB	23.90 ± 11.07 21.95	3.86 ± 5.28 1.61	5.49	< 0.001*
CD11c	2.34 ± 1.51 2.28	13.03 ± 7.20 14.65	5.37	< 0.001*
Foxp3	3.35 ± 3.10 2.86	14.22 ± 8.45 17.55	3.84	< 0.001*

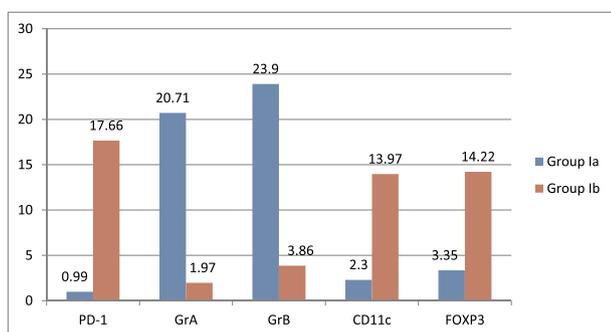


Fig. 2b. comparison of gene expression levels between two patients' subgroups.

12%.The IPIwas zero in 30%, 1 in 26%, 2 in 40% and 3 in 4% of patients (Table 2).

ThePD-1, FOXP3,GrA, GrB and CD11c gene expressions were significantly increased in DLBCL patients than controls (Table 3and Fig. 2a).

Table 5
Correlation between different gene expressions inpatients group.

	PD-1		GrA		GrB		CD11c		FOXP3	
	r	P	r	p	r	p	r	P	r	P
PD-1	-	-	-0.26	0.007*	-0.32	0.001*	0.636	< 0.001	0.44	< 0.001*
GrA	-0.26	0.007*	-	-	0.63	< 0.001*	-0.654	< 0.001	-0.009	0.928
GrB	-0.32	0.001*	0.63	< 0.001*	-	-	-0.557	< 0.001	-0.30	0.002*
CD11C	0.636	< 0.001	-0.654	< 0.001	-0.557	< 0.001	-	-	0.519	< 0.001
FOXP3	0.44	< 0.001*	-0.009	0.928	0.30-	0.002*	0.519	< 0.001	-	-

Table 6
COX survival regression of NHLpatients.

	Overall survival		
	Hazard ratio	95% CI	P value
Age	0.952	0.89–1.01	0.149
Extranodal site	0.433	0.01–9.76	0.599
IPI score	19.28	2.04–181.78	0.010*
Staging	11.63	0.48–277.64	0.130
B2 microglobulin level	9.11	0.52–156.9	0.128
LDH (IU/L)	0.985	0.97–0.99	0.011*
PD-1	1.30	1.03–1.63	0.024*
GrA	0.705	0.33–1.50	0.365
GrB	0.955	0.71–1.26	0.030*
CD11C	0.98	0.97–0.99	0.043*
FOXP3	0.801	0.50–1.08	0.04*

Significant difference was present between the two subgroups of lymphoma patients(1aand1b) regarding relative quantitative (RQ) gene expressions of PD-1, CD11c and FOXP3 (increase with advanced stages III and IV).While regarding GrA and GrB genes there was significant decrease with advanced stages III and IV (Table 4 and Fig. 2b).

Regarding RQ of PD-1 gene expression, there was a significant negative correlation between it and each of GrA and GrB gene expressions. Also, there was a significant positive correlation between it and each of CD11c and FOXP3 gene expressions (Table 5).

There was a significant positive correlation between GrA and GrB gene expressions with significant negative correlation between each of them and FOXP3 gene expressions. There was significant positive correlation between FOXP3 and CD11c gene expressions (Table 5).

IPI score, LDH levels and expression of PD-1, FOXP3, GrB and CD11c genes are independent risk factors for the overall survival (OS) in DLBCL patients, while age, staging, B2microglobulin levels and expression of GrA gene are dependent risk factors (Table 6).

4. Discussion

Emerging studies clear that tumor microenvironment (TME) has great importance. It plays a double role. As, It can both inhibit tumor growth by either killing cancer cells or suppressing their growth, It also enhance tumor progression either by providing conditions that activate tumor growth or selecting the tumor cells which are fit for survival [18].

Regarding diffuse large B-cell lymphoma (DLBCL), the lymph node microenvironment, containing components affect the growth of lymphoma, as T cells, growth factors, dendritic cells, chemokines and stromal cells [19].

Programmed cell death-1 (PD-1), is a member of the CD28 superfamily which is highly expressed on the surface of activated T lymphocytes and dendritic cells in a different types of cancers or immune diseases [20].

PD-1 is an immune checkpoint and guards against autoimmunity through apoptosis of antigen-specific T-cells, this prevents autoimmune diseases, but it can also prevent the immune system from killing cancer cells. So, immune tolerance to the malignant lymphoma occurs as a

result of increased PDL-1 expression, which leads to suppression of the T-cell response [21].

This study revealed that FOXP3 and PD-1 genes showed over expression in patients with DLBCL. Also their expressions increased with tumor aggressiveness (staging). FOXP3 and PD-1 gene expressions were independent factors associated with the overall survival (OS). Thus they can be considered as new immunological targeting for treatment of NHL.

Cancer cells can avoid and suppress immune responses through activation of Blocking the activities of inhibitory immune checkpoint proteins, like PD-1, PD-L1, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and Foxp3+ Tregs restoring T cell function, has considered as breakthrough therapies against cancer, render lethal cancers into treatable disease [22–24].

In our study we correlated between the expressions of both PD-1 and FOXP3 genes. They were up regulated and over expressed in advanced cases (stage III and IV) with significant positive correlation with each other.

Matthew et al., 2014 confirmed the positive correlation between numbers of FOXP3 and PD1 expressing CD4⁺ T-cells as well as between total CD4⁺ T-cells and each of these subsets in DLBCL [19].

The association between FOXP3 expression in tumor cells and prognosis of cancer patients is debatable, as the association with both poor and good prognosis has been reported in various types of cancers. However, most results have reported a positive relationship between the expression of FOXP3 with cancer metastasis and clinical outcome [25–27].

The most potent inducers of apoptosis are GrA and GrB, members of a family of serine proteases known as granzymes (granule-associated enzymes), which cleave specific intracellular substrates to initiate distinct cell death routes in a target cell [28,29].

The current study showed that there was significant increase in the expression of GrA and GrB genes in DLBCL with significant decrease in advanced stages (group1b). Also there were negative correlations between their expressions and each of PD-1 and FOXP3 gene expressions. Because FOXP3 and GrB are likely to identify functionally distinct populations of immunosuppressive and activated cytotoxic effector lymphocytes, the relative proportion of these subsets and their relationship to patient outcome was also examined by Todd W. Kelley et al, 2017 and the analysis indicated that the ratio of FOXP3+ T cells to GrB+ T/NK cells was highly correlated with outcome. Cases with ratios of more than 1 had significantly longer FFS and OS in Hodgkin lymphoma [30].

Souza et al., 2017 stated that quantitative real time PCR showed that patients with NHL had over expression of perforin and granzyme B (GrB) transcripts compared to the controls. Also, patients who had expression of both genes below the median found for the NHL group had lower survival rates. So, they believe that perforin and granzyme B are potential prognostic markers in NHL [31].

Cancers of both lymphoid and non-lymphoid origin also express GrB inducing apoptosis in transformed cells, and in some cases this expression is linked to pathogenesis or cell death. For example, GrB expression in urothelial carcinoma was implicated in promoting tumour cell invasion, whereas its expression in nasal-type NK/T lymphomas was found to correlate with increased apoptosis [32].

While, Granzyme-A-induced cell death is mainly characterized by generation of single-stranded DNA nicks, rather than the oligonucleosomal DNA fragments typical of granzyme-B-induced apoptosis. Moreover, GrA acts in a caspase-independent manner to induce a cell death exhibiting all the features of apoptosis [33]. Mice lacking components of the granule-induced death mechanism are more prone to tumours. For example, perforin-deficient mice develops spontaneous B-cell lymphomas and cannot control tumour growth in experimental settings [34].

The role of granzymes in tumour clearance as a Cancer escape mechanisms is controversial. Several intracellular inhibitors of granzymes are reported to be overexpressed in tumour cells [35,36].

This study also revealed significant increase in CD11c gene expression in DLBCL patients with significant decrease in advanced stages. Also there was significant positive correlation between its expression and FOXP3 gene expression.

Dendritic cells (DCs) play a key role in the induction of adaptive immune responses. When activated, it cause regulation of certain molecules on their surface that share in T lymphocyte activation. Blood contains at least two distinct DC types, the myeloid DCs (mDCs) and the plasma DCs [37,38].

CD11c is a dendritic cell marker, which induces a cytotoxic effect on lymphoma cells. It is often considered a marker for mDCs. Thus CD11c is important for regulating immune responses, but it is also expressed by a subpopulation of human NK cells. The relationship between mDCs and cancer prognosis is unclear [39].

Lee, Seul, et al (2017) revealed that patients with CD11c expressing DLBCL had a significantly better OS rate than those without, and both CD11c and FOXP3 expressions can predict the tumor outcome beside to IPI score and extranodal site [37]. This is similar to our results which found that CD11c, GrB, PD-1 and FOXP3 expressions, LDH levels and IPI score retained independent prognostic significance on the overall survival (OS).

5. Conclusion

Patients with DLBCL have variable PD-1, FOXP3, GrA, GrB and CD11c gene expressions levels, which are correlated with the overall survival (OS) indicating that they can be good predictors of outcome in these patients.

Conflict of interest

All authors have no conflict of interest.

Author contribution statement

Adel Nassar and Abd ElMonem Eltorgoman design of the work; Eman Badr and Nesreen G Elhelbawy, done the lab investigation and wrote the main manuscript; Safa M saber help in interpretation of data; All authors reviewed the manuscript.

Declaration of competing interest

The author(s) declare no competing financial and non financial interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrep.2020.100731>.

1. The authors mentioned that PD-L1 is involved in apoptosis of tumor specific T cells however it is overexpressed in patients, can authors explain?
2. In subjects and methods, the authors should add the start and end date of the study specifically they added a survival course of disease.
3. In introduction, authors should not start paragraph with As and so either remove as or complete the previous paragraph(As, genetic alterations of genes that control cell differentiation, proliferation, and survival are also required).
4. Authors should remove this consent from methods as it is previously mentioned in subjects (After taking informed written consent from all subjects and approval of the Ethical Committee of Medical Research- Menoufia Faculty of Medicine)
5. Authors mentioned that they did CBC in methods however there is no data nor results in manuscript, is it non significant or can authors explain?

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