Cancer Management and Research

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ORIGINAL RESEARCH

PD-L1 and miR-34a are Prognostic Factors for Primary Gastric Diffuse Large B-Cell Lymphoma Patients Treated with R-CHOP

This article was published in the following Dove Press journal:
Cancer Management and Research

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Introduction: Primary gastric diffuse large B-cell lymphoma (GDLBCL) is a heterogeneous disease in clinicopathological features and prognosis. Programmed death ligand-1 (PD-L1) and microRNA-34a (miR-34a) play crucial roles in GDLBCL progress. The purpose of this research is to explore the clinical significance of PD-L1 and miR-34a expression in GDLBCL.

Patients and Methods: The expressions of PD-L1 and miR-34a were examined by IHC and qRT-PCR in 109 patients who were diagnosed with GDLBCL and were treated with rituximab plus cyclophosphamide, doxorubicin, prednisone vincristine and prednisone chemotherapy (R-CHOP) from January 2010 to December 2018.

Results: PD-L1 level was significantly higher in tumor tissues than adjacent non-tumor tissues (60.5%, $P<0.001$), while the miR-34a level was just reversed (50.5%, $P<0.001$), which was negatively correlated ($r=-0.524$, $P<0.001$). Notably, PD-L1-positive and miR-34a-negative expressions were significantly correlated with the advanced Lugano stage of IIE-IV stage ($P<0.001$ and $P<0.01$), elevated serumal LDH levels ($P<0.001$ and $P<0.05$), B symptoms present ($P<0.001$ and $P<0.001$), non-GCB subtype ($P<0.001$ and $P<0.001$) and negative Bcl-2 expression ($P<0.05$ and $P<0.001$). PD-L1 high and miR-34a low expression groups had more patients with IPI scores of 2 or greater ($P<0.001$ and $P<0.05$) and poor R-IPI ($P<0.01$ and $P<0.01$). The complete response rate was upregulated in patients with negative PD-L1 and positive miR-34a expression after R-CHOP treatment.

Discussion: PD-L1 expression and miR-34a expression were significantly associated with clinicopathological characteristics and survival prognosis; they may serve as novel prognostic markers in GDLBCL patients who were treated with R-CHOP. Immunotherapies targeting PD-L1 and miR-34a pathway may have therapeutic potential in GDLBCL.

Keywords: GDLBCL, R-CHOP, PD-L1, miR-34a, tumor immunotherapy

Introduction

The gastrointestinal tract is commonly accompanied by non-Hodgkin lymphoma (NHL), and the stomach is the major site to be affected by 60% NHL patients with digestive tract involvement.[1] Among lymphomas of the stomach, diffuse large B-cell lymphoma (DLBCL) and mucosa-associated lymphoid tissue lymphoma (MALT) are the two major types.[2] Chemotherapy is recommended as the first-line treatment for GDLBCL according to the NCCN Guidelines and the Japanese gastric cancer treatment guidelines 2010 (ver. 3). Surgery is recommended as an urgent and palliative treatment for patients presenting with obstruction, bleeding, or severe perforation.[3] The

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http://doi.org/10.2147/CMAR.S247874

therapy of R-CHOP has obviously improved the therapeutic effects of GDLBCL patients, though the number of benefits patients is limited. Rituximab plus first-line chemotherapy treatment of GDLBCL reduces the incidence of central nervous system relapses.[4] Due to the significant clinical and biological heterogeneity of GDLBCL patients, more efficient models or prognostic factors are needed to classify patients with various survival outcomes. Targeted therapies such as antibodies against programmed cell death 1 (PD-1) and its ligand (PD-L1) have a great prospect in the treatment of different malignant tumors.[5] Recently, many clinical retrospective studies have exhibited responses to PD-1 antibodies for patients whose PD-L1 is expressed in tumor cells or tumor-infiltrating immune cells.[6]

MicroRNAs (miRNAs) are a group of noncoding RNAs with about 22 nucleotides, which can downregulate the target genes' expression through changing translational efficiency and stability of the target mRNAs.[7] Currently, in the human genome, there are 1400 human miRNAs identified nearly, and each miRNA may influence many target genes. MiRNAs are expressed in a tissue-specific manner and produce an effect on cell differentiation, apoptosis, and proliferation. Recent trials have hinted that the abnormal expression of miRNAs is related to the occurrence of cancer. The members of the miR-34 family are regulated transcriptionally by p53, which are downregulated in acute myeloid leukemia and chronic lymphocytic leukemia.[8,9] MiR-34a acts as a tumor suppressor by promoting apoptosis in some tumors.[10] Interestingly, the upregulated PD-L1 level is tightly linked to the downregulated miR-34a level in lung cancer and B-cell lymphomas,[11,12] and PD-L1 has been hinted to be a regulatory target of miR-34a.[13] In this research, we demonstrated that PD-L1 was upregulated and miR-34a was downregulated in GDLBCL tissues. Through this research, we aimed to determine the prognostic implications of PD-L1 and miR-34a levels for clinical GDLBCL.

Patients and Methods

Clinical Specimens

From January 2010 to December 2018, a total of 109 patients that pathologically diagnosed with primary GDLBCL at Hunan Cancer Hospital were recruited for this study. Specimens used for qRT-PCR were preserved in liquid nitrogen. Samples used for IHC were fixed with formalin and embedded into paraffin. The diagnosis was established according to the histopathological and immunohistochemical criteria in the 2016 WHO classification system. The R-CHOP was administered every three weeks: day 1, rituximab (375 mg/m^2); day 2, vincristine (1.4 mg/m^2, maximal dose 2 mg), doxorubicin (50 mg/m^2), and cyclophosphamide (750 mg/m^2); day 2 to 6, prednisone (100 mg/day). It was administered for three to eight cycles. After R-CHOP was done, interventional field radiotherapy (IFRT) was supplied for extranodal disease, residual disease, and/or previous mass disease. The number of chemotherapy cycles and the adjustments of regimen dose were determined by physicians. The International Working Group Recommendations for Response Criteria with non-Hodgkin's lymphoma was used to assess treatment response.[14] Retrospective research was made according to each patient's medical records.

qRT-PCR

The TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA in tissue samples according to the manufacturer's instruction. qRT-PCR for PD-L1 and miR-34a was performed using the ABI PRISM 7700 instrument (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) with gene-specific primers and the SYBR Green (Takara, Tokyo, Japan). In brief, 1 μg total RNA was reverse-transcribed into cDNA in a total volume of 20 μL, and 1 μL cDNA was used as a template for qRT-PCR. Specific primers used for qRT-PCR are listed in Table 1. GAPDH and U6 were set as the internal control of mRNA and miRNA, respectively.[15] The relative level of PD-L1 and miR-34a was calculated by the $2^{-\Delta\Delta Ct}$ method. All experiments were independently repeated three times. The effect of miR-34a level in cancer tissues on prognosis was determined by Kaplan-Meier analysis according to the median.

Table 1 Primer Pairs Used for qRT-PCR

Genes	Primer Sequences
GAPDH	FP: 5′-GCACCGTCAAGGCTGAGAAC-3′ RP: 5′-TGGTGAAGACGCCAGTGGA-3′
PD-L1	FP: 5′-AGATCAAAGAGAGCCTGCGG-3′ RP: 5′-AGGGGTCCTCCTTCAGGG-3′
RNU6	FP: 5′-GCGCGTCGTGAAGCGTTC-3′ RP: 5′-GTGCAGGGTCCGAGGT-3′
miR-34a	FP: 5′-CGGTATCATTTGGCAGTGTCT-3′ RP: 5′-GTGCAGGGTCCGAGGT-3′

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PD-L1, programmed death ligand-1; RNU6, U6 snRNA; miR-34a, microRNA-34a; FP, forward primer; RP, reverse primer.

Immunohistochemistry (IHC)

The 5-μm paraffin-embedded tissue section was dewaxed in xylene and rehydrated in graded alcohols. Antigens were retrieved in boiled 1 μM sodium citrate solution (pH = 6.0) for two minutes. After blockade with normal goat serum for one hour, slices were sequentially incubated with the primary antibody anti-PD-L1 (PD-L1 antibody purchased from Proteintech) overnight at 4°C and the secondary antibody at room temperature for one hour, stained with DAB solution and counterstained with hematoxylin. Tissue slices were viewed at 400× magnification under inverted microscopy, and represented images were represented in figures. Three fields per section were analyzed.

We defined the criterion of PD-L1 positivity that tumor cells were positively stained by more than 5%. Two observers scored 20% cases to assess reproducibility. Cases were supposed to be evaluable that more than a quarter of tissue was available for morphologic analysis and more than one positively staining tumor-infiltrating macrophage as a positive internal control.

Immunostaining for CD5, CD10, CD20, Bcl-2 and Bcl-6 with antigen retrieval and antibody dilutions on paraffin sections was performed according to manufacturers' recommendations and previous report elsewhere.[16]

Follow-Up

The information of follow-up was gained from the patients or patients' relatives and information systems of our hospital that contained progression-free survival (PFS), overall survival (OS), and overall response rate (ORR). PFS was defined from the date of the pathological diagnosis to progression. OS was defined from the date of pathological diagnosis to death or the last follow-up. The response to first-line therapy consisted of complete remission (CR), partial response (PR), stable disease (SD), or progressive disease (PD). ORR was defined as CR plus PR. The patient's prognosis was analyzed.

Statistical Analysis

The normality of data distribution was tested by the Kolmogorov–Smirnov test. Pearson's chi-square test, independent-samples *t*-test, paired-samples *t*-test, Pearson's correlation, nonparametric Mann–Whitney U or Wilcoxon signed ranks test, Spearman's rank correlation and logistic regression were used when appropriate. A univariate test and multivariate test were used to look for the influence of each clinical variable on prognosis. Data analysis was performed using SPSS version 22.0 (SPSS, Inc., Chicago, IL, USA). All data were mean ± SD or median (range). *P<0.05, **P<0.01, ***P<0.001 were considered statistically significant.

Results

Clinicopathological Characteristics of GDLBCL

Of the 109 patients in this study, the primary clinicopathologic characteristics were shown in Table 2. The median age was 53.7 years old (range 18–76 years), and 62.4% of patients were less than 60 years. Few patients (9.2%) had an unfavorable performance status (PS) (PS≥2). 67 patients were at Lugano stage I or II2, while the other 42 patients were at stage IIE or IV. Next, there were 4 patients (3.7%) in a high-risk group, 22 patients (20.2%) in a high-intermediate risk group, 23 patients (21.1%) in a low-intermediate risk group, and 60 patients (55.1%) in the low-risk group, according to International Prognostic Index (IPI) scores. What's more, 49 patients (45%) were Bcl-2 positive, while 60 patients (55.1%) were Bcl-2 negative.

PD-L1 Was Highly Expressed and miR-34a Was Lowly Expressed in GDLBCL

The expression level of PD-L1 mRNA and miR-34a was determined by qRT-PCR in 109 tumor specimens with GDLBCL. According to the median of miR-34a expression level, patients were divided into low-level and high-level groups. MiR-34a was lowly expressed in 54 specimens (49.5%), and significant difference (0.391 vs. 0.842, P<0.001) was uncovered between the low-level and high-level groups (Figure 1A, the right part). Moreover, a significantly negative correlation (Pearson's correlation, r= −0.524, P<0.001) existed between PD-L1 and miR-34a level (Figure 1B). Among 109 GDLBCL cases, 15 patients were surgically treated. PD-L1 mRNA level in cancer tissues of those 15 specimens was higher than that in paired noncancerous gastric tissues (1.694 vs. 1.000, P<0.001, Figure 1C), while the miR-34a level was just reversed (0.619 vs. 1.000, P<0.001, Figure 1D).

We also examined the PD-L1 protein level by IHC in 109 FFPE specimens with GDLBCL. We defined the criterion of PD-L1 positivity that tumor cells were positively stained more than 5% (Figure 1E and F). As a result, PD-L1 was highly expressed in 66 specimens (60.6%), and the relative level of PD-L1 mRNA in the IHC-positive group

Table 2 Clinical Features of 109 Patients with GDLBCL According to PD-L1 and miR-34a Expression

Groups	Clusters	N (%)	PD-L1			miR-34a		
			-	+	P	-	+	P
Age	≤60 >60	68 (62.4%) 41 (37.6%)	28 15	40 26		33 21	35 20	
Gender	Male Female	52 (47.7%) 57 (52.3%)	21 22	31 35		26 28	26 29	
BMI	≤25 >25	88 (81.7%) 21 (18.3%)	35 8	53 13		42 12	46 9	
Hs-CRP	≤6mg/l >6mg/l	90 (82.6%) 19 (17.4%)	36 7	54 12		45 9	45 10	
ECOG PS	0–1 ≥2	99 (90.8%) 10 (9.2%)	40 3	59 7		48 6	51 4	
Lugano stage	I–II2 IIE-IV	67 (61.5%) 42 (38.5%)	37 6	30 36	***	26 28	41 14	**
Serumal LDH	Normal Elevated	57 (52.3%) 52 (47.7%)	33 10	24 42	***	23 31	34 21	*
Extranodal site	0–1 ≥2	91 (83.5%) 18 (16.5%)	38 5	53 13		42 12	49 6	
B symptoms	No Yes	57 (52.3%) 52 (47.7%)	34 9	23 43	***	18 36	39 16	***
IPI	0–1 ≥2	60 (55.0%) 49 (45.0%)	34 9	26 40	***	23 31	37 18	*
R-IPI	0 1–2 3–5	21 (19.3%) 62 (56.9%) 26 (23.9%)	18 20 5	3 42 21	**	4 32 18	17 30 8	**
Pathology	GCB Non-GCB	45 (41.3%) 64(58.7%)	29 14	16 50	***	13 41	32 23	***
CD5	- +	99(90.8%) 10 (9.2%)	39 4	60 6		51 3	48 7	
CD10	- +	68 (62.4%) 41 (37.6%)	30 13	38 28		29 25	39 16	
Bcl-2	- +	60 (55.0%) 49 (45.0%)	18 25	42 24	*	39 15	21 34	***
Bcl-6	- +	40 (36.7%) 69 (63.3%)	19 24	21 45		18 36	22 33	

Notes: -: Negative; +: Positive. Statistically significant, *p<0.05,**p<0.01, ***P<0.001.

Abbreviations: GDLBCL, gastric diffuse large B-cell lymphoma; PD-L1, programmed death ligand-1; miR-34a, microRNA-34a; BMI, body mass index; Hs-CRP, high-sensitivity C-reactive protein; ECOG PS, Eastern Cooperative Oncology Group Performance Status; LDH, lactate dehydrogenase; IPI, International Prognostic Index; R-IPI, revised International Prognostic Index; GCB, germinal center B-cell.

was significantly higher than that in the negative group (2.147 vs. 1.001, P<0.001) (Figure 1A, the left part). And significant Spearman's rank correlation was observed between PD-L1 protein level determined by IHC and mRNA level examined by qRT-PCR (ρ= 0.712, P<0.001, Table 3).

Correlations Between the Expression of PD-L1, miR-34a, and Clinicopathological Features

To characterize the clinical role of PD-L1 and miR-34a in GDLBCL furtherly, we tried to precisely identify the correlations of PD-L1 and miR-34a level with clinicopathological indicators, such as patient gender, age, BMI, Hs-CRP, ECOG PS, Lugano stage, serumal LDH, extranodal site tumor number, B symptoms, IPI, R-IPI, pathology subtype and expression level of CD5, CD10, Bcl-2, and Bcl-6. And no significant correlations between the expression level of PD-L1, miR-34a, and the main clinical features such as patient gender, age, BMI, Hs-CRP, ECOG PS, extranodal site tumor number, CD5, CD10 and Bcl-6 (Table 2). However, high level of PD-L1 and low level of miR-34a exhibited a high incidence with advanced Lugano stage (Stage IIE and IV, P<0.001 and P<0.01, respectively), elevated serumal LDH levels (P<0.001 and P<0.05, respectively), B symptoms present (P<0.001 and P<0.001, respectively), non-GCB subtype (P<0.001 and P<0.001, respectively) and negative Bcl-2 expression (P<0.05 and P<0.001, respectively). Furthermore, PD-L1 high-level and miR-34a low-level groups had more patients with IPI scores of 2 or greater (P<0.001 and P<0.05, respectively) and poor R-IPI (P<0.01 and P<0.01, respectively).

Relationships Between Treatment Outcomes and Expression Level of PD-L1 and miR-34a

Table 4 showed the response details to first-line treatment (R-CHOP±IFRT). Following initial therapy, the ORR was 82.6%, and CR was achieved in 72 patients (66.1%), but 27 patients (24.8%) showed disease progression or relapses subsequently. Notably, PD-L1 and miR-34a levels were significantly correlated with both CR efficacy and progression/relapse rate. The PD-L1-positive group had a lower CR rate and a higher progression/relapse rate than the negative group (CR rate, 32.1% vs. 33.9%, P=0.005; progression/relapse rate, 20.2% vs. 4.6%, P=0.010; Table 4). Moreover, the miR-34a-positive group had a higher CR rate and a lower progression/relapse rate than the miR-34a-negative group (CR rate, 40.4% vs. 25.7%, P=0.015; relapse/progression rate, 6.4% vs. 18.3%, P=0.003; Table 4). In order to exclude

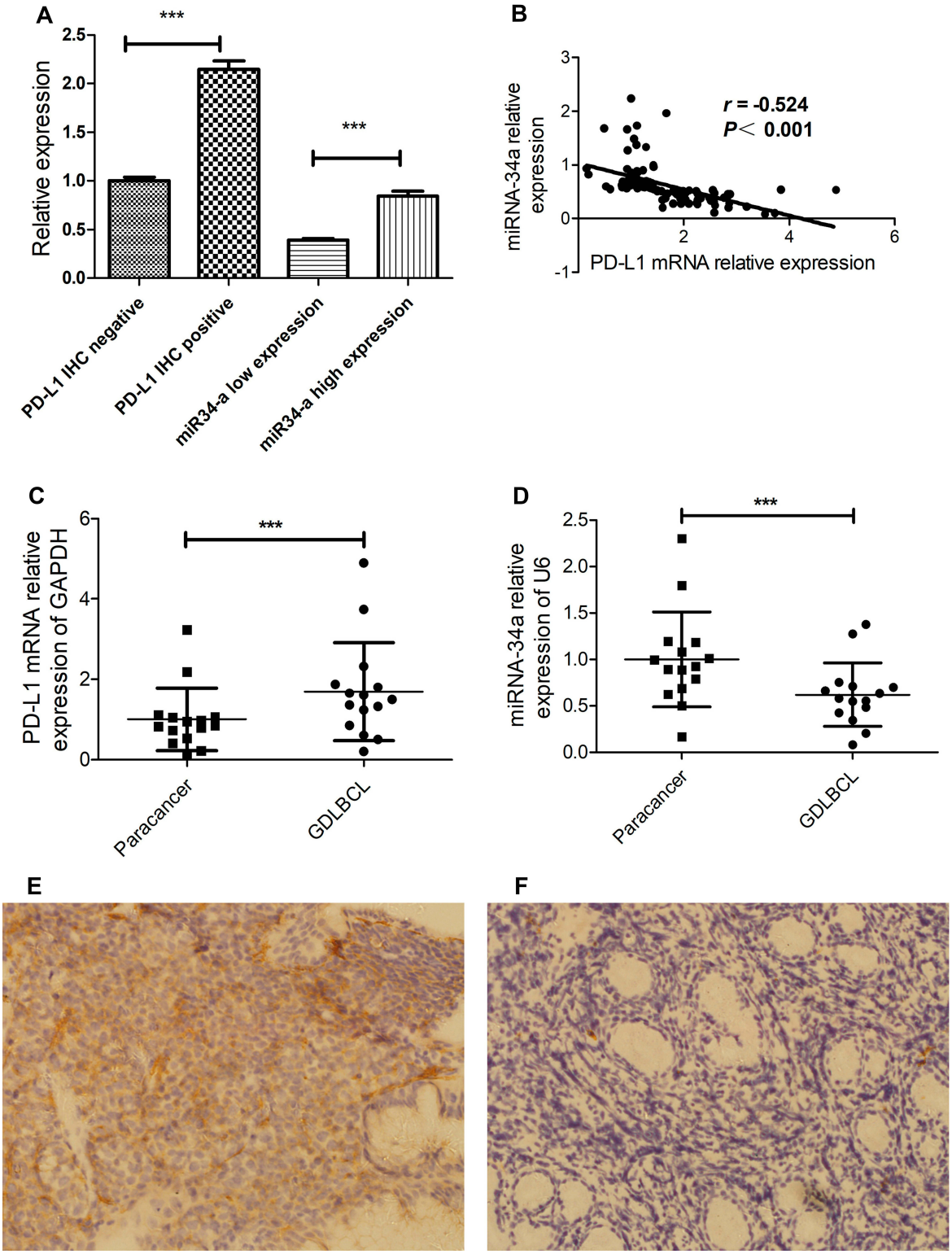

Figure 1 PD-L1 is overexpressed and miR-34a is low expressed in GDLBCL tissues. (**A**) the left part, the relative expressions of PD-L1 mRNA in the IHC-positive group were higher than in IHC-negative group; the right part, the relative expressions of miR-34a mRNA in the high expression group were higher than in low expression group. GAPDH and U6 were set as the internal control of mRNA and miRNA, respectively. Statistically significant, ***P<0.001. (**B**) Pearson's correlation analysis showed that there was significant correlation between relative expression of PD-L1 and miR-34a expression. GAPDH and U6 were set as the internal control of mRNA and miRNA, respectively. (**C**) PD-L1 mRNA expression was obviously higher than that in adjacent noncancerous gastric tissues. GAPDH was set as the internal control of mRNA. Statistically significant, ***P<0.001. (**D**) miR-34a expression was obviously lower than that in adjacent noncancerous gastric tissues. U6 was set as the internal control of miRNA. Statistically significant, ***P<0.001. (**E**) PD-L1 is highly expressed in GDLBCL tissues (× 400). (**F**) PD-L1 was weakly expressed in GDLBCL tissues (× 400).
Abbreviations: IHC, immunohistochemistry; PD-L1, programmed death ligand-1; miR-34a, microRNA-34a; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RNU6, U6 snRNA; GDLBCL, primary gastric diffuse large B-cell lymphoma.

Table 3 Relationship Between PD-L1 Protein Level and mRNA Level

Groups	No. of Patients	PD-L1 mRNA	ρ#	P
PD-L1 IHC negative	44	1.001 ± 0.2610	0.712	<0.001
PD-L1 IHC positive	66	2.147 ± 0.685		

Note: #Spearman's rank correlation.
Abbreviations: PD-L1, programmed death ligand-1; IHC, immunohistochemistry.

Table 4 Comparison of Treatment Outcomes Based on PD-L1 and miR-34a Expression Level

Features	N (%)	PD-L1			miR-34a		
		-	+	P	-	+	P
Response				0.005			0.015
CR	72 (66.1%)	37	35		28	44	
PR	18 (16.5%)	3	15		13	5	
SD	6 (5.5%)	1	5		5	1	
PD	13 (11.9%)	2	11		8	5	
Progression/relapse				0.010			0.003
No	82 (75.2%)	38	34		34	48	
Yes	27 (24.8%)	5	22		20	7	
Treatment modality				0.079			0.176
R-CHOP	94 (86.2%)	34	60		49	45	
R-CHOP+IFRT	15 (13.8%)	9	6		5	10	

Notes: -: Negative; +: Positive.
Abbreviations: PD-L1, programmed death ligand-1; miR-34a, microRNA-34a; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; R-CHOP, rituximab plus cyclophosphamide, doxorubicin, vincristine, and Prednisone; IFRT, involved-field radiotherapy.

possible treatment-related bias, we compared the treatment type with the expression of PD-L1 and miR-34a. Patients treated with R-CHOP alone and patients treated with R-CHOP+IFRT were applied for this comparison, whereas no significant difference between PD-L1- and miR-34a-negative and -positive groups (P=0.079 and P=0.176, respectively; Table 4).

Survival Analysis and Follow-Up

During an average follow-up of 56 months (range: 2.7–87.8 months), there were 38 deaths (34.9%). Comparison of patient's survival outcomes between the negative and positive PD-L1 groups, the positive PD-L1 expression group showed inferior PFS and OS (P=0.004 and 0.001, respectively; Figure 2A and B). Besides, the negative miR-34a expression group showed inferior PFS and OS when compared with the miR-34a-positive group (P=0.009 and 0.009, respectively; Figure 2C and D).

Univariate Analysis and Multivariate Analysis of Prognostic Factors

So as to identify the potential significant prognostic factors, a univariate analysis of each main factor was performed concerning the prognosis of GDLBCL patients. The hazard ratio and P value of each factor were used to predict the difference in prognosis. Then, a multivariate Cox proportional hazards model was used to determine the significance of each factor. Through univariate analysis, factors were gradually included in the model, which determined that the significant prognostic factors in PFS and OS of GDLBCL patients were PD-L1, miR-34a, Lugano stage, presence of B symptoms, IPI and Bcl-2 (Table 5). The results of the multivariate analysis indicated that PFS could be predicted based on PD-L1, miR-34a, and IPI (Table 5), and that OS could be predicted based on PD-L1 and miR-34a (Table 6).

Discussion

There are some breakthroughs in cancer diagnosis and chemotherapy. Recently, immunotherapies targeting PD-1/PD-L1 have become effective strategies for the treatment of some types of tumors.[5] In this research, PD-L1-negative expression and miR-34a-positive expression were favorable prognostic impacts for GDLBCL patients. Another notable finding of this research was that the low expression of PD-L1 was associated with the high expression of miR-34a. Recent research demonstrates that immune checkpoint PD-L1 is regulated by miR-34a in DLBCL.[12] Hence, careful consideration of PD-L1 and miR-34a in GDLBCL may be important for selecting PD-1/PD-L1 checkpoint blockades.

There are limited trials on the predictive value of PD-L1 in GDLBCL, and most of which are retrospective studies with a controversial conclusion. A lot of previous studies reveal that PD-L1 upregulation is linked to an unfavorable prognosis. However, some investigators find no prognostic significance based on PD-L1 expression, and the others demonstrate that PD-L1 is a beneficial prognostic in GDLBCL.[17] In previous studies, the cut-off values of PD-L1 positivity are varied from 5% to 30%, while the percentages of PD-L1-positive DLBCL are varied from 11% to 75%.[18] This heterogeneity shows that even experienced hematopathologists can hardly distinguish between PD-L1-positive cells and PD-L1-negative cells. Recently, Chen et al[19] reported the prevalence of

PD-L1-positive DLBCL, in which the use of PD-L1/Pax5 double staining in selected cases may reduce the heterogeneities. However, it is still subjective and difficult to promote worldwide. In our study, qRT-PCR was used to detect PD-L1 mRNA expression levels, and IHC was used to detect PD-L1 protein levels. The relative expression of PD-L1 mRNA in the IHC-negative group was lower than in IHC-positive group. There was a significant correlation (Spearman's rank correlation, ρ= 0.712, P<0.001) between PD-L1 protein level and mRNA level. Thus, we might use the qRT-PCR method to get more accurate PD-L1 expression, instead of IHC.

Our study revealed a significant correlation between PD-L1-positive expression and non-GCB, which was similar to former research.[18] Besides, we found that positive PD-L1 expression was linked to Bcl-2 expression significantly. And the increased PD-L1 level was usually related to poor clinical features, such as B symptoms and IPI scores of 2 or greater. PD-L1 expression induces worse OS irrespective of using rituximab.[20] Therefore, GDLBCL patients with high PD-L1 expression do not benefit from first-line treatment.[21] In literature reports, tumor cells that express PD-L1 have many mechanisms to escape T-cell immunity.[22] PD-1/PD-L1 pathway induces apoptosis of PD-1$^+$ tumor-associated antigen-specific T cells, which is one of its important mechanisms.[23] Another possible reason is that chemotherapeutic resistance may arise from positive PD-L1 expression partly, which may link to poor prognosis.[21] These findings suggest that PD-L1 expression may be favorable to discover GDLBCL patients who have a high disease progression risk.

MiRNAs can affect lineage choice or critical developmental checkpoints in the hematopoietic process.[24] MiR-34a is one of the tumor suppressor miRNAs, which is the key

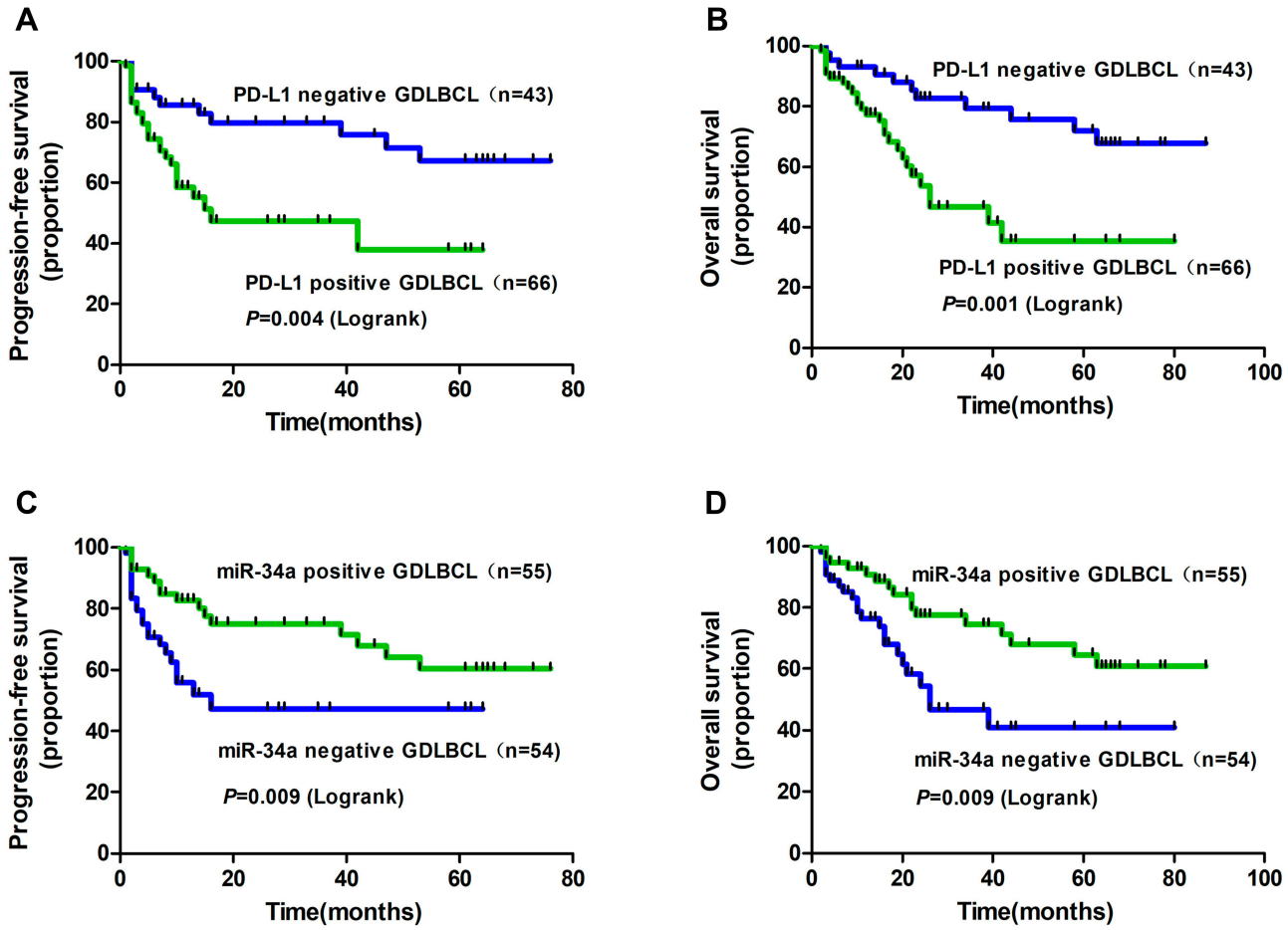

Figure 2 Comparison of survival outcome according to PD-L1 and miR-34a expression. (**A**) Patients with positive PD-L1 expression showed significant inferior PFS. (**B**) Patients with positive PD-L1 expression showed significantly inferior OS. (**C**) Patients with negative miR-34a expression showed significant inferior PFS. (**D**) Patients with negative miR-34a expression showed significantly inferior OS.

Abbreviations: PD-L1, programmed death ligand-1; miR-34a, microRNA-34a; GDLBCL, primary gastric diffuse large B-cell lymphoma; PFS, progression-free survival; OS, overall survival.

Table 5 Univariable Analysis of Clinicopathological Prognostic Factors for PFS and OS in GDLBCL Patients

Risk Factor	PFS			OS		
	HR	95% CI	P	HR	95% CI	P
PD-L1	2.841	1.368–5.901	0.005	3.211	1.546–6.672	0.002
miR-34a	0.426	0.220–0.825	0.011	0.422	0.218–0.818	0.011
Age	1.424	0.751–2.701	0.280	1.552	0.818–2.943	0.179
Gender	1.125	0.593–2.134	0.718	1.095	0.578–2.075	0.781
BMI	1.762	0.828–3.751	0.141	1.515	0.710–3.233	0.283
Hs-CRP	0.825	0.345–1.974	0.665	0.849	0.355–2.032	0.849
ECOG PS	1.907	0.743–4.893	0.180	1.708	0.666–4.381	0.265
Lugano stage	2.043	1.067–3.913	0.031	1.794	0.944–3.410	0.074
Serumal LDH	1.500	0.786–2.863	0.218	1.533	0.805–2.917	0.194
Extranodal site	1.569	0.689–3.574	0.284	1.087	0.478–2.471	0.842
B symptoms	2.195	1.138–4.232	0.019	2.446	1.264–4.730	0.008
IPI	2.772	1.428–5.378	0.003	2.556	1.327–4.927	0.005
R-IPI	1.567	0.970–2.533	0.067	1.430	0.905–2.260	0.125
Pathology	1.954	0.980–3.896	0.057	1.916	0.962–3.819	0.064
CD5	1.636	0.682–3.920	0.270	1.709	0.713–4.094	0.229
CD10	1.003	0.512–1.965	0.993	0.947	0.484–1.852	0.873
Bcl-2	0.504	0.257–0.987	0.046	0.549	0.279–1.079	0.082
Bcl-6	1.483	0.746–2.947	0.261	1.512	0.762–3.001	0.237

Abbreviations: HR, hazard ratio; CI, confidence interval; PFS, progression-free survival; OS, overall survival; PD-L1, programmed death ligand-1; miR-34a, microRNA-34a; BMI, body mass index; Hs-CRP, high-sensitivity C-reactive protein; ECOG PS, Eastern Cooperative Oncology Group Performance Status; LDH, lactate dehydrogenase; IPI, International Prognostic Index; R-IPI revised International Prognostic Index.

Table 6 Multivariate Analysis of Clinicopathological Prognostic Factors for PFS and OS in GDLBCL Patients

Risk Factor	PFS			OS		
	HR	95% CI	P	HR	95% CI	P
PD-L1	2.277	1.030–5.034	0.042	1.360	1.169–5.583	0.029
miR-34a	0.502	0.280–0.961	0.045	0.506	0.285–0.957	0.048
B symptoms	1.078	0.484–2.403	0.855	1.487	0.679–3.259	0.321
IPI	2.234	1.008–4.591	0.048	1.872	0.866–4.047	0.111

Abbreviations: HR, hazard ratio; CI, confidence interval; PFS, progression-free survival; OS, overall survival; PD-L1, programmed death ligand-1; miR-34a, microRNA-34a; IPI, International Prognostic Index.

regulatory factor of a tumor suppressor. It is downregulated in some kinds of tumors and participates in the occurrence and development of tumors.[10] It is reported that the constitutive expression of miR-34a blocked the development of B cells in the transitional period from pre-B cells to pre-B cells, resulting in a decrease of mature B cells.[10] As a direct activator of p53, miR-34a is involved in the p53 network. Wild type p53 lead miR-34a transcription, and miR-34a targets a variety of molecules involved in cell transformation and carcinogenesis.[10,24] Usually, miR-34a is often downregulated in some kinds of tumors.[12] In our research, we found that the expression of miR-34a was decreased in GDLBCL than that in adjacent noncancerous gastric tissues, aligning with previous viewpoints.[15] Our results also found that low expression of miR-34a was linked to non-GCB subtype and Bcl-2, and was usually related to poor clinical features, such as B symptoms and IPI scores of 2 or greater. Previous research shows that the expression of miR-34a is downregulated in GDLBCL and may be related to the patient's progression and metastasis by targeting Bcl-2, which has been confirmed as a regulatory target of miR-34a.[15] Moreover, the miR-34a low expression is linked to the worse outcome of GDLBCL. The expression level of miR-34a is reduced in a non-GCB type of DLBCL cells and tumor tissues. The overall survival rate of patients with lower miR-34a is worse, while increased expression of miR-34a makes non-GCB DLBCL cells respond to doxorubicin treatment.[25] These results suggested that miR-34a was an important

tumor suppressor and a potential biomarker for treatment and prognostic of GDLBCL.

Recent research reveals that miR-34a regulates immune checkpoint PD-L1 in lung cancer and B-cell lymphomas.[11,12] In our research, we found that the low level of PD-L1 was significantly linked to a high level of miR-34a. And patients with PD-L1 high expression and miR-34a low expression had a high incidence of advanced Lugano stage (IIE or IV), elevated serumal LDH levels, B symptoms present, non-GCB subtype, and negative Bcl-2 expression. What's more, the PD-L1 high expression and miR-34a low expression groups were linked to IPI scores of 2 or greater and poor R-IPI. Whereas the PD-L1-negative expression and miR-34a-positive expression were favorable prognostic factors in GDLBCL. What are the reasons or mechanisms that such down-regulation or up-regulation of these biomarkers influenced prognosis? The following reasons may relate to this question. Firstly, miR-34a functions as a tumor suppressor gene and links the p53 network by FOXP1 and Bcl-2.[15] Additionally, PD-L1 expression is regulated by p53 via miR-34a.[12] In keeping with these, PD-L1 and miR-34a were uncovered to be independent prognostic factors for PFS and OS in GDLBCL patients treated with R-CHOP in our study.

In summary, our results show that approximately one-half of GDLBCL patients are positive for PD-L1 and negative for miR-34a. Positive PD-L1 expression and negative miR-34a expression are linked to a high prevalence of elevated IPI scores and elevated LDH levels. Positive PD-L1 expression and negative miR-34a expression are also linked to high progression/relapse rates and low CR rates after the first-line treatment of R-CHOP. PD-L1 and miR-34a are independent prognostic factors for PFS and OS in GDLBCL patients treated with R-CHOP. While it needs more trials to confirm our findings and to better understand the biological functions of PD-L1 and miR-34a in GDLBCL. The PD-L1-positive and miR-34a-negative patients are not rare in GDLBCL but have a worse clinical prognosis. In contrast, low PD-L1 and high miR-34a expression are identified to be a favorable prognostic group, though their prognostic impact could be different in some GDLBCL. This problem should be further settled in future research to choose superior candidates as PD-1/PD-L1 checkpoint blockades.

Consent to Participate and Ethics Approval

All tissue specimens used in this study were obtained from Hunan Cancer Hospital with informed consent from patients. All procedures followed in experiments were following the ethical commission of Hunan Cancer Hospital and were complied with the Declaration of Helsinki.

Acknowledgments

This study was supported by Industry Research Project from the Development and Reform Commission of Hunan Province (2015-99, to Chaohui Zuo), and Key Research Project from the Hunan Provincial Science and Technology Department (2017k2130, to Chaohui Zuo). We thank Chen Liu for assistance with the writing of the manuscript.

Disclosure

Chaohui Zuo is currently investigating the clinical treatment and molecular biology of the recurrence and metastasis for digestive oncology as a researcher. Yajun Li is currently investigating the clinical treatment and molecular biology of the recurrence and metastasis for digestive lymphoma as a researcher. The authors report no other conflicts of interest in this work.

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