Clinics in Oncology

Research Article
Published: 27 Oct, 2021

Expression of Serum Amyloid A and Misfolded Transthyretin Protein in Relapsed/Refractory Diffuse Large B cell Lymphoma and its Clinical Significance

*Shu-Ling Hou**

Department of Lymphatic Oncology, Shanxi Bethune Hospital, China

Abstract

Background: This study aims to evaluate the prognostic value of Serum Amyloid A (SAA) and misfolded Transthyretin (TTR) in Relapsed/Refractory Diffuse Large B-Cell Lymphoma (R/R-DLBCL).

Methods: A total of 50 DLBCL patients were included in the present study. Among these patients, 30 patients had R/R-DLBCL, 20 patients had remission/stabilization DLBCL, and 10 patients had chronic lymphadenitis. The SELDI technique, Tris-Tricine Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (Tris-Tricine-SDS-PAGE), and shotgun-LTQ-MS method were used to determine and identify the protein of SAA and TTR in R/R-DLBCL. The bioinformatics technique was used to determine the structure and function of the protein. The clinical feature data were statistically analyzed using SPSS 21.0 software, and chi-square test, Kaplan-Meier curve and log-rank test ware used.

Results: A molecular weight of approximately 12,000 Da and 14,000 Da were found in serum of R/R-DLBCL in protein finger graphics and Tris-Tricine-SDS-PAGE. Then, these were identified as SAA and TTR. The high expression of SAA and TTR protein (SAA+TTR+) was significantly associated with the extranodal lesion, and high level LDH and NCCN-IPI scores (P=0.017, P=0.017 and P=0.008, respectively), and correlates with the non-GCB type (the Φ correlation coefficient was 0.538) in R/R-DLBCL patients. Furthermore, the high expression of TTR protein (TTR+) was significantly associated with high levels of LDH, extranodal lesion C-MYC expression and non-GCB in R/R-DLBCL patients (The P-value was 0.028, P=0.003, the Φ correlation coefficients were 0.305 and 0.385, respectively) and the high expression of SAA protein (SAA+) was significantly associated with B-symptoms, high level of LDH and non-GCB in R/R-DLBCL patients (The P-values were 0.02, 0.011, Φ correlation coefficient was 0.390, respectively). The survival time of the SAA+ group, TTR+ group and SAA+TTR+ group were shorter than that of the negative group (P=0.001, P=0.034 and P=0.003, respectively). Multivariate analysis showed that LDH levels were an independent risk factor of poor prognosis (P<0.05).

Conclusion: Both the SAA and misfolded TTR were poor prognosis factors for R/R-DLBCL.

Keywords: Serum Amyloid A; Misfolded transthyretin; Diffuse large B-cell lymphoma; Relapsed/refractory

OPEN ACCESS

Correspondence:
Shu-Ling Hou, Department of Lymphatic Oncology, Shanxi Bethune Hospital, Taiyuan 030032, China, E-mail: 13834134457@139.com

Received Date: *26 Sep 2021*
Accepted Date: *18 Oct 2021*
Published Date: *27 Oct 2021*

Citation:
Shu-Ling Hou. Expression of Serum Amyloid A and Misfolded Transthyretin Protein in Relapsed/Refractory Diffuse Large B cell Lymphoma and its Clinical Significance. Clin Oncol. 2021; 6: 1871.

ISSN: 2474-1663

Abbreviations

TTR: Transthyretin; R/R-DLBCL: Relapsed/refractory diffuse large B-cell lymphoma; SAA+TTR+: SAA and TTR protein; SAA: Serum Amyloid A; DLBCL: Diffuse large B-cell lymphoma; GCB: Germinal Center B-cell-like; IHC: Immunohistochemistry; WHO: World Health Organization; OS: Overall Survival; CR: Complete Response; PR: Partial Remission; NHL: Non-Hodgkin's Lymphoma; PFS: Progression-Free Survival; ABC: Activated B-Cell

Introduction

Diffuse Large B-Cell Lymphoma (DLBCL) is a group of non-Hodgkin's lymphomas with significant heterogeneity in morphology, genetics, molecular biology and pathological features. DLBCL patients presently use the standard R-CHOP protocol. Rituximab is an anti-CD20 monoclonal antibody that has efficacy in patients with indolent and aggressive forms of B-cell Non-Hodgkin's Lymphoma (NHL), which can be consider as a standard first-line treatment option when combined

with cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) chemotherapy in patients with R/R DLBCL. However, approximately 50% of patients continue to have poor treatment [1]. There are several prognostic factors of DLBCL: (1) Patient-related factors: age and physical status; (2) Tumor-related factors: Stage, tumor burden, proliferation score, and extranodal involvement; (3) Invasive indicators and related factors: β2 Microglobulin (β2-MG), and serum Lactate Dehydrogenase (LDH) levels. Due to low sensitivity or specificity, the prognosis cannot be accurately predicted. Furthermore, 30% to 40% of patients who received initial treatment is refractory or relapses after initial remission [2]. At present, these two groups of patients could not be identified at the time of diagnosis. If relapsed/refractory can be identified before treatment, and a stronger intervention is adopted early, the cure rate of DLBCL will be increased. Furthermore, at present, no biomarkers can predict R/R-DLBCL. In 2007, the lymphoma team of Shanxi Dayi Hospital applied Surface Enhanced Laser Desorption Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF-MS) technology, and found that an M/Z 11,000 Da to 12,000 Da protein peak appeared before death. On this basis, the protein was isolated and identified as Serum Amyloid A (SAA), and it was found that this was accompanied by misfolded Thyroprotein (TTR). The present study aimed to investigate SAA and misfolded TTR in DLBCL, and the expression and its relationship with the patient's clinical features and prognosis.

Materials and Methods

Patient data collection

Grouping design: From March 2013 to September 2015, 50 patients with complete data in the Department of Lymphatic Oncology, Shanxi Dayi Hospital were enrolled into the present study. Among these patients, 30 patients with R/R-DLBCL were assigned to the study group (18 male patients and 12 female patients, 3:2), 20 DLBCL patients (10 male patients and 10 female patients, 1:1) and 10 chronic lymphadenitis patients (five male patients and five female patients, 1:1) with remission/stabilization were assigned to the control group, and 30 healthy subjects (15 male patients and 15 female patients, 1:1) were assigned to the negative control group.

Inclusion criteria:

(1) Comply with DLBCL pathological diagnostic criteria;

(2) Meet the relapsed/refractory diagnostic criteria: Relapsed DLBCL refers to new lesions in newly diagnosed patients after chemotherapy. Refractory DLBCL can be diagnosed by one of the following criteria: i) Tumor volume reduction <50% or disease progression after four courses of standard chemotherapy; ii) Standard chemotherapy to CR but recurrence within six months. iii) Relapsed for ≥ 2 after CR. iv) recurrence after hematopoietic stem cell transplantation [2].

Exclusion criteria:

(1) Pregnant woman;

(2) <14 years old and >80 years old;

(3) HIV positive;

(4) Combined with other tumors to be treated;

(5) Acute infection concurrent;

Patient details: Among the 30 patients with R/R-DLBCL, 25 patients were non-specific, four patients had the special subtype, and one patient had gray lymphoma (between DLBCL and Hodgkin's lymphoma). Among the four special subtypes, one case had rich T-cell/tissue cell large B-cell lymphoma, while three cases had mediastinal DLBCL. From the disease site, 15 cases of primary extranodal DLBCL were divided as follows: Three cases of tonsil, three cases of nasal cavity, two cases of thyroid, two cases of testis, two cases of stomach, one case of mammary gland, one case of adrenal gland, and one case of oropharynx.

Furthermore, among the cases, eight cases (26.7%) were <60 years old, while 22 cases (73.3%) were ≥ 60 years old. **Ann Arbor clinical stage:** Nine cases were in the early stage (two cases were in stage I, while seven cases were in stage II; 30%), while 21 cases were in the late stage (4 cases were in stage III, while 17 cases were in stage IV; 70%). **B symptoms:** 20 cases (66.7%) had B symptoms, while 10 cases (33.3%) had no B symptoms. Furthermore, 1 case (3.3%) was in the low risk group and had an NCCN-IPI score of 0 to 1 points, 8 cases (40%) were in the low-risk group and had an NCCN-IPI score of 2 to 3 points, 18 cases (40%) were in the high-risk group and had an NCCN-IPI score of 4 to 5 points, and 3 cases (16.7%) were in the high-risk group and had an NCCN-IPI score of ≥ 6 points. **Extranodal lesions:** 15 cases (50.0%) had lesions in the lymph nodes, while 15 cases (50.0%) had lesions in the extranodal; 15 cases (50.0%) had increased serum lactate dehydrogenase, while 15 cases (50.0%) had normal serum lactate dehydrogenase; serum albumin was normal in eight cases (26.7%), while serum albumin decreased in 22 cases (73.3%); the ECOG physical status score was 0 to 2 points in 22 cases (73.3%) and 3 to 5 points in eight cases (26.7%).

Sample collection: For the case group and control group, 3 ml of fasting venous whole blood was collect in the morning before treatment, and the supernatant was extracted and stored in a freezer at -80°C.

Treatment: Patients with double/three strikes were treated with R-EPOCH as the initial treatment plan. The remaining patients were treated with R-CHOP as the initial treatment plan. Relapsed/refractory patients were rescued by Hyper-CVAD, GDP, ICE, DICE and other programs.

Prognostic evaluation indicators: Overall Survival (OS) refers to the time from the start of treatment to any cause of death.

Efficacy evaluation: All newly diagnosed patients were evaluated for efficacy after four cycles of chemotherapy [3]. The efficacy was divided, as follows: Complete Response (CR), Partial Remission (PR), Stable (SD), and Progression (PD).

Follow-up: All patients were followed up by clinic or telephone. The starting point was March 2013, and the follow up was up to January 2018. The median survival time was 20.5 months (range: 1 to 58 months), and there was no loss of follow-up.

Reference standard: The 2016 World Health Organization (WHO) lymphoid hematopoietic tumor pathological typing system was used to identify the pathological diagnosis. The Hans typing system was used to determine whether it was a Germinal Center B-cell-like (GCB) or non-GCB subtype. The protein expression of C-MYC, BCl-2, BCl-6 and Ki67 was determined using the Immunohistochemistry (IHC) method to determine whether there was double/triple expression. The Ann Arbor clinical staging system was used. The NCCN-IPI scoring system was used to evaluate the prognosis index, and the physical status was determined using the ECOG score. The R-EPOCH regimen was selected for patients with

double/triple expression. The other patients were treated using the R-CHOP regimen. All relapsed/refractory patients have rescued a regimen.

Immunohistochemistry

IHC staining was performed on all patients. Due to the lack of tissue samples in some patients, a full set of Hans typing and double/triple expression staining were not performed. For 20 patients with complete IHC data, Hans classification was performed: 7 were GCB subtype and 13 were non-GCB subtype.

Double/triple expression: BCl-2 protein was detected in 21 cases. Among these cases, 16 cases were positive for ≥ 50%, while five cases were positive for <50%. Furthermore, BCl-6 protein was detected in 23 cases, including 16 positive cases and seven negative cases. C-MYC protein was detected in 18 cases, including positive for ≥ 40% in 13 cases and positive for <40% in five cases. Ki67 protein was detected in 27 cases, including positive for ≥ 70% in 22 cases and positive for <70% in five cases. C-MYC and BCl-2 protein were expressed in nine cases, while C-MYC, BCl-2 and BCl-6 protein were expressed in four cases. Due to the Double/triple expression immunohistochemical staining were retrospective diagnosis, some patients had insufficient specimens and could not perform a full set of C-MYC, BCl-2 and BCl-6 markers. Patients with complete IHC data for analysis: 5 cases of double-expression (BCL-2+C-MYC) patients and 4 cases of triple-expression patients (BCL-2+C-MYC+BCl-6).

Protein fingerprinting and SELDI-TOF-MS

A protein fingerprinting instrument (Ciphergen, USA) provided the wcx2 chip, pbs-iic chip identification machine, and protein chip software. The reagent was manufactured by Sigma (USA).

The SELDI-TOF-MS technique was used to determine the protein fingerprint of the patients, Tris-Tricine-SDS-PAGE was used to determine the differential peptides, and serum shot-LTQ-MS was used to identify the differential peptides. Gel pieces were cut from SDS PAGE, destained with 30% ACN/100 mM NH_4HCO_3 until the gels were destained. The gels were dried in a vacuum centrifuge. The in-gel proteins were reduced with dithiothreitol (10 mM DTT/100 mM NH4HCO3) for 30 min at 56°C, then alkylated with iodoacetamide (200 mM IAA/100 mM NH_4HCO_3) in the dark at room temperature for 30 min. Gel pieces were briefly rinsed with 100 mM NH_4HCO_3 and ACN, respectively. Gel pieces were digested overnight in 12.5 ng/μl trypsin in 25 mM NH_4HCO_3. The peptides were extracted three times with 60% ACN/0.1% TFA. The extracts were pooled and dried completely by a vacuum centrifuge. And bioinformatics technology was also applied. The proteins were analyzed to identify and determine the structure and function.

Statistical analysis

Statistical analysis was performed using the SPSS 21.0 software system. Chi-square test was used to analyze clinical count data, the Kaplan-Meier curve was used for the survival analysis, and Log-Rank test was used to compare single-factor survival differences. Cox regression was used for the multivariate analyses. A P-value <0.05 was considered statistically significant.

Results

Determination, electrophoresis and identification of serum protein

SELDI-TOF-MS in patients with relapsed/refractory DLBCL

The SELDI-TOF-MS assay revealed that there were two peaks of differential protein fingerprints in the serum of patients with R/R-DLBCL (Figure 1): (A) a molecular weight of approximately 11 kDa to 12 kDa; (B) a molecular weight of approximately 14 kDa (Figure 1).

Tris-Tricine-SDS-PAGE protein electrophoresis results: five patients with R/R-DLBCL had five differential protein bands, when compared to patients in the remission/stabilization phase, patients with chronic lymph node inflammation, and healthy subjects. Two high expression: A band: The molecular weight was approximately 12,000 Da; B band: The molecular weight approximately 14,000 Da, the molecular weight was basically the same as that determined by SELDI-TOF-MS. Three low expression, high expression in the control group, C band: The molecular weight was approximately 10,000 Da; D band: The molecular weight of approximately 15,000 Da to 20,000 Da; E band: The molecular weight of approximately 15,000 Da (Figure 1).

Shotgun-LTQ-MS identification results: band a: Serum Amyloid A (SAA); band b: degraded or misfolded Transthyretin (TTR) (Figure 2).

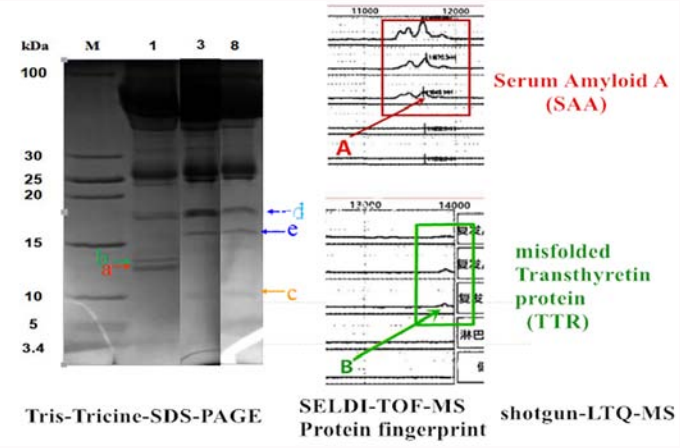

Figure1: Left: Tris-Tricine-SDS-PAGE results for relapsed/refractory DLBCL patients (lane 1), patients in remission/stable phase (lane 3), and patients with chronic lymph node inflammation (lane 8). There were 5 differential protein bands, including 2 up regulated bands in relapsed/refractory DLBCL patients: A (12 kDa) and b (14 kDa), and 3 down regulated bands: c (10 kDa), d (15~20 kDa) and e (15 kDa). M: Small molecular weight protein Markers. Right: Two differential protein fingerprint peaks in the serum of patients with relapsed/refractory DLBCL. A: Serum amyloid A (SAA1/2), 11-12 kDa; b: Degraded or misfolded Transthyretin (TTR), 14 kDa. Identified with SELDI-TOF-MS and Shotgun-LTQ-MS.

	Total number of peptides	Unique number of peptides	Coverage	Theoretical molecular weight	Theoretical isoelectric point	Protein Login ID and name
b	31-48	9	69.39%	15887.03	5.52	sp|P02766|TTHY_HUMAN Transthyretin OS=Homo sapiens GN=TTR PE=1 SV=1
	31-48	9	69.39%	15887.03	5.52	tr|E9KL36|E9KL36_HUMAN Transthyretin OS=Homo sapiens GN=HEL111 PE=2 SV=1
	32-48	9	55.43%	20198.86	5.16	tr|A6XGL1|A6XGL1_HUMAN Transthyretin OS=Homo sapiens PE=2 SV=1
a	41-53	9	60.66%	13527.14	9.2	sp|P0DJI9|SAA2_HUMAN Serum amyloid A-2 protein OS=Homo sapiens GN=SAA2 PE=1 SV=1
	41-51	8	63.93%	13562.11	6.28	tr|D3DQX7|D3DQX7_HUMAN Serum amyloid A protein OS=Homo sapiens GN=SAA1 PE=3 SV=1
	42-49	8	63.93%	13532.08	6.28	sp|P0DJI8|SAA1_HUMAN Serum amyloid A-1 protein OS=Homo sapiens GN=SAA1 PE=1 SV=1

Figure 2: Shotgun-LTQ-MS identification and bioinformatics identification results.

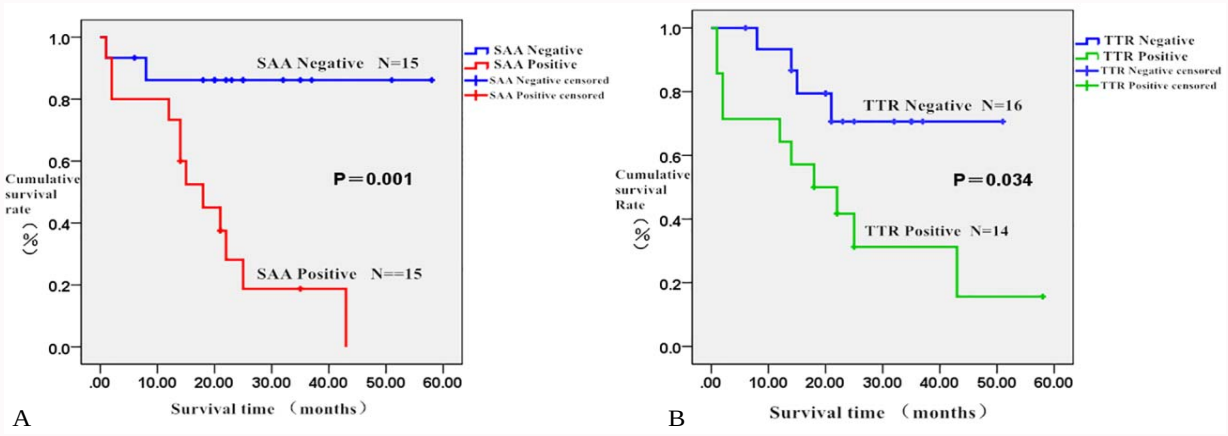

Figure 3: Comparison of survival between SAA+ and TTR+ groups in R/R-DLBCL.
A: In the 30 patients with R/R-DLBCL, there was a significantly shorter survival in group of SAA+ (15/30) compared with group of SAA- (15/30) (P=0.001).
B: In the 30 patients with R/R-DLBCL, there was a significantly shorter survival in group of TTR+ (14/30) compared with group of TTR- (16/30) (P=0.034).

The relationship of the expression of SAA and misfolded TTR in R/R-DLBCL with clinical/pathological features

The experimental electropherogram revealed that the SAA and misfolded TTR had a high differential expression in the R/R-DLBCL group, but not in the remission/stabilized DLBCL group, chronic lymphasitic node group and healthy subjects (Figure 1).

The high expression of SAA protein (SAA+) was significantly associated with B-symptoms, high level of LDH and non-GCB in R/R-DLBCL patients (The P-values were 0.02, 0.011, Φ correlation coefficient was 0.390, respectively). However, there was no significant correlation between SAA+ and the age, gender, Ann Arbor stage, extranodal lesions, ECOG score, Hans classification, albumin, NCCN-IPI scores, C-myc, bcl-2, bcl-6, Ki67, and double/triple expression (P>0.05) (Table 1, 2).

The high expression of TTR protein (TTR+) was significantly associated with high levels of LDH, extranodal lesion C-MYC expression and non-GCB in R/R-DLBCL patients (The P-value was 0.028, P=0.003, the Φ correlation coefficients were 0.305 and 0.385, respectively). There was no correlation between TTR+ and age, gender, Ann Arbor stage, B symptoms, ECOG score, albumin, NCCN-IPI scores, BCL-2, BCL -6, Ki67, and double/triple expression (P>0.05) (Table 1, 2).

SAA and TTR expression and the prognostic value of patients with DLBCL

In the 30 patients with R/R-DLBCL, SAA+15 (15/30) and SAA-15 (15/30), the SAA+ group had a significantly shorter survival, when compared with the SAA-group (P=0.001, Figure 3A).

In the 30 patients with R/R-DLBCL, TTR+14 (14/30) and TTR-16 (16/30), the survival was significantly shorter in the TTR+ group than that in the TTR-group (P=0.034, Figure 3B).

Correlation analysis between high expression of SAA and TTR protein and the clinical and pathological features

The high expression of SAA and TTR protein (SAA+TTR+) was significantly associated with extranodal lesion, high levels of LDH, and NCCN-IPI scores in patients with R/R-DLBCL (The P-value was 0.017; P=0.017 and P=0.008, respectively). There was no significant correlation between SAA+TTR+ and the age, gender, B symptoms, Ann Arbor stage, ECOG score, and albumin level of the patient

Table 1: Correlation between expression of SAA and TTR in R/R-DLBCL and clinical features.

Clinical features	N	TTR		χ^2	*P*	SAA		χ^2	*P*
		TTR-(%)	TTR+ (%)			SAA-(%)	SAA+ (%)		
Gender									
male	18	10 (55.6)	8 (44.4)	0.09	0.77	9 (50.0)	9 (50.0)	<0.001	1
Female	12	6 (50.0)	6 (50.0)			6 (50.0)	6 (50.0)		
Age									
<60	8	6 (75.0)	2 (25.0)	1.04	0.31	3 (37.5)	5 (62.5)	0.17	0.68
≥ 60	22	10 (45.5)	12 (54.5)			12 (54.5)	10 (45.5)		
Stage									
I	2	1 (50.0)	1 (50.0)	1.22	0.75	2 (100.0)	0 (0.0)	4.613	0.26
II	7	5 (71.4)	2 (28.6)			4 (57.1)	3 (42.9)		
III	4	2 (50.0)	2 (50.0)			3 (75.0)	1 (25.0)		
IV	17	8 (47.1)	9 (52.9)			6 (35.3)	11 (64.7)		
B symptoms									
no	10	7 (70.0)	3 (30.0)	0.82	0.37	8 (80.0)	2 (20.0)	5.4	0.02
yes	20	9 (45.0)	11 (55.0)			7 (35.0)	13 (65.0)		
Extranodal lesion									
no	15	4 (26.7)	11 (73.3)	8.57	0	6 (63.6)	9 (36.4)	1.2	0.27
yes	15	12 (80.0)	3 (20.0)			9 (47.4)	6 (52.6)		
NCCN-IPI									
Low risk group	1	1 (100.0)	0 (0.00)	4.58	0.21	1 (100.0)	0 (0.0)	3.128	0.37
Low-medium risk group	8	6 (75.0)	2 (25.0)			5 (62.5)	3 (37.5)		
Medium High risk group	18	7 (38.9)	11 (61.1)			7 (38.9)	11 (61.1)		
High risk group	3	2 (66.7)	1 (33.3)			2 (66.7)	1 (33.3)		
ECOG									
5-Mar	8	3 (37.5)	5 (62.5)	0.4	0.53	2 (25.0)	6 (75.0)	1.534	0.22
0-2	22	13 (59.0)	9 (41.0)			13 (59.1)	9 (40.9)		
Serum albumin									
≥ 40 g/L	8	5 (62.5)	3 (37.5)	0.04	0.85	5 (62.5)	3 (37.5)	0.17	0.68
<40 g/L	22	11 (50.0)	11 (50.0)			10 (45.5)	12 (54.5)		
LDH									
<250 IU/L	15	11 (73.3)	4 (26.7)	4.82	0.03	11 (73.3)	4 (26.7)	6.533	0.01
≥ 250 IU/L	15	5 (33.3)	10 (66.7)			4 (26.7)	11 (73.3)		

(Table 3).

SAA+TTR+ correlates with the patient's non-GCB (the Φ correlation coefficient was 0.538), but was not associated with BCL-2, C-MYC, BCL-6, Ki67, and double/triple expression (Table 4).

The prognostic value of patients with SAA, and TTR protein high expressed in DLBCL

In the 30 patients with R/R-DLBCL, the SAA+TTR+ (9/30 patients) in the SAA+TTR+ group had a significantly shorter survival time, when compared to the SAA-TTR- (P=0.003), SAA+TTR-, and SAA-TTR+ group (Figure 4).

Univariate and multivariable analysis of the impact of prognostic indicators on survival in patients with R/R-DLBCL

The median survival time of 30 patients with R/R-DLBCL was 20.5 months, and the 3-year total OS rate was 53.3%. B symptoms, extranodal lesions, SAA+, TTR+, SAA+TTR+, LDH levels, ECOG scores, and NCCN-IPI scores had significant effects on the prognosis (P=0.008, P=0.017, P=0.001, P=0.034, P=0.003, P<0.001, P<0.001, and P=0.02), while for age, gender, Ann Arbor stage, Hans classification, BCL-2, C-MYC, BCL-6, ki67 level, double expression, triple expression, and serum albumin level, the effect on the prognosis was not significant (P>0.05) (Table 5). Then B symptoms, extranodal lesions, SAA+, TTR+, SAA+TTR+, LDH levels, ECOG scores, and NCCN-IPI scores were enrolled for multivariate analysis. Multivariate analysis showed that LDH levels were an independent risk factor of poor prognosis (P<0.05) (Table 5, 6).

Discussion

DLBC is the most common type of NHL (Non-Hodgkin lymphoma), accounting for 30% to 40% of newly diagnosed NHL every year in the world, and the percentage of invasive lymphoma is 80% [1]. Approximately 50% of patients with DLBCL are R/R-DLBCL but only 10% of them would be recovered. Thus, R/R-DLBCL act as one of the main bottle-necks in DLBC treatment. At present,

Table 2: Correlation between SAA and TTR in R/R-DLBCL with pathological features.

Pathological features	N	TTR- (%)	TTR+ (%)	φ	P	SAA- (%)	SAA+ (%)	φ	P
Hans classification									
GCB	7	6 (85.7)	1 (14.3)	0.385	0.09	5 (71.4)	2 (28.6)	0.39	0.08
non-GCB	13	6 (46.2)	7 (53.8)			4 (30.8)	9 (69.2)		
BCL-2									
<50%	5	3 (60.0)	2 (40.0)	0.032	0.88	1 (20.0)	4 (80.0)	-0.36	0.1
≥ 50%	16	9 (56.2)	7 (43.8)			10 (62.5)	6 (37.5)		
C-MYC									
<40%	5	4 (80.0)	1 (20.0)	0.305	0.2	3 (60.0)	2 (40.0)	0.055	0.81
≥ 40%	13	6 (46.2)	7 (53.8)			7 (53.8)	6 (46.2)		
BCL-6									
negative	7	5 (71.4)	2 (28.6)	0.086	0.68	5 (71.4)	2 (28.6)	0.255	0.22
Positive	16	10 (62.5)	6 (37.5)			7 (43.8)	9 (56.3)		
ki67									
<70%	5	2 (40.0)	3 (60.0)	-0.19	0.33	3 (60.0)	2 (40.0)	0.078	0.69
≥ 70%	22	14 (63.6)	8 (36.4)			11 (50.0)	11 (50.0)		
C-MYC/BCL-2									
Non-double expression	25	13 (52.0)	12 (48.0)	0.107	0.74	11 (44.0)	14 (56.0)	2.16	0.14
Double expression	5	3 (60.0)	2 (40.0)			4 (80.0)	1 (20.0)		
C-MYC/BCL-2/BCL-6									
Non-three expression	26	15 (57.7)	11 (42.3)	0.223	0.22	13 (50.0)	13 (50.0)	0	1
Three expression	4	1 (25.0)	3 (75.0)			2 (50.0)	2 (50.0)		

Table 3: Correlation between SAA+ TTR+ and clinical features in R/R DLBCL.

Clinical features	N	SAA and TTR		X^2	*P*	Clinical features	N	SAA and TTR		X^2	*P*
		Non-SAA+ TTR+ (%)	SAA+ TTR+ (%)					Non-SAA+ TTR+ (%)	SAA+ TTR+ (%)		
Gender						LDH					
male	18	13 (72.2)	5 (27.8)	<0.001	1	<250 IU/L	15	14 (93.3)	1 (6.7)	5.714	0.017
Female	12	8 (66.7)	4 (33.3)			≥ 250 IU/L	15	7 (46.7)	8 (53.3)		
Age						Serum albumin					
<60	8	7 (87.5)	1 (12.5)	0.657	0.42	≥ 40 g/L	8	7 (87.5)	1 (12,5)	0.657	0.417
≥ 60	22	14 (63.6)	8 (36.4)			<40 g/L	22	14 (63.6)	8 (36.4)		
B symptoms						Extranodal lesion					
no	10	5 (50)	5 (50)	1.607	0.21	no	15	7 (46.7)	8 (53.3)	5.714	0.017
Yes	20	16 (80)	4 (20)			yes	15	14 (93.3)	1 (6.7)		
ECOG						Serum albumin					
5-Mar	8	4 (50)	4 (50)	0.982	0.32	≥ 40 g/L	8	7 (87.5)	1 (12,5)	0.657	0.417
0-2	22	17 (77.2)	5 (22.8)			<40 g/L	22	14 (63.6)	8 (36.4)		
Stage						NCCN-IPI					
I	2	2 (50.0)	0 (50.0)	0.37	0.83	Low risk group	1	1 (100.0)	0 (0.0)	11.669	0.008
II	7	5 (71.4)	2 (28.6)			Low-medium risk group	8	8 (100.0)	0 (0.0)		
III	4	4 (50.0)	0 (50.0)			medium High school risk group	18	9 (50.0)	9 (50.0)		
IV	17	10 (47.1)	7 (52.9)			High risk group	3	3 (100.0)	0 (0.0)		

the diagnosis treatment for relapsed/refractory DLBCL remains a problem that is presently being worked on. The prognostic factors related to DLBCL, from clinical manifestations, can be divided into (1) patient-related factors: age and physical status; (2) tumor-related factors: staging, tumor burden, proliferation score, extranodal involvement; (3) invasive index-related factors: β2 microspheres Protein (β2-MG), Lactate serum Dehydrogenase (LDH) levels. The international prognostic factors of DLBCL are generally recognized as NCCN-IPI (International Prognostic Index), which includes five factors: Age, lactate dehydrogenase, physical fitness status, extranodal

Table 4: Correlation between SAA+ TTR+ and pathological features in R/R DLBCL.

Pathological features	N	Non-SAA+ TTR+ (%)	SAA+ TTR+ (%)	φ	P	Pathological features	N	non-SAA+ TTR+ (%)	SAA+ TTR+ (%)	φ	P
Hans classification						BCL-2					
GCB	7	7 (100)	0 (0.0)	0.54	0.02	<50%	5	4 (80.0)	1 (20.0)	0.106	0.63
non-GCB	13	6 (46.2)	7 (53.8)			≥ 50%	16	11 (68.8)	5 (31.2)		
C-MYC						BCL-6					
<40%	5	4 (80.0)	1 (20.0)	0.11	0.65	negative	7	5 (71.4)	2 (28.6)	-0.04	0.86
≥ 40%	13	9 (69.2)	4 (30.8)			Positive	16	12 (75.0)	4 (25.0)		
C-MYC/BCL-2						C-MYC/BCL-2/BCL-6					
Non-double expression	25	17 (68.0)	8 (32.0)	0.29	0.59	Non-three expression	26	19 (73.1)	7 (26.9)	0.171	0.35
Double expression	5	4 (80.0)	1 (20.0)			Three expression	4	2 (50.0)	2 (50.0)		
ki67											
<70%	5	4 (80.0)	1 (20.0)	0.06	0.74						
≥ 70%	22	16 (72.7)	6 (27.3)								

Table 5: Single factor survival analysis (log-rank) affecting the prognosis of patients with R/R-DLBCL.

Clinical/pathological features	Number of cases	χ^2	*P value*	Clinical/pathological features	Number of cases	χ^2	*P value*
Age				ki67			
<60	8	1.81	0.178	<70%	5	1.188	0.276
≥ 60	22			≥ 70%	22		
Gender				TTR			
male	18	0.79	0.373	negative	16	4.484	0.034*
female	12			Positive	14		
Stage				SAA			
I	2	7.12	0.068	negative	15	10.75	0.001*
II	7			Positive	15		
III	4			SAA and TTR			
IV	17			SAA-TTR-	10	13.79	0.003*
B symptoms				SAA+TTR-	6		
no	10	6.96	0.008*	SAA-TTR+	5		
yes	20			SAA+TTR+	9		
Extranodal lesion				Serum albumin			
no	15	5.65	0.017	≥ 40 g/L	8	2.314	0.128
yes	15			<40 g/L	22		
Hans classification				LDH			
GCB	7	0.94	0.333	<250 IU/L	15	15.62	<0.001*
non-GCB	13			≥ 250 IU/L	15		
BCL-2				ECOG			
<50%	5	0.39	0.532	5-Mar	8	13.34	<0.001*
≥ 50%	16			0-2	22		
C-MYC				NCCN-IPI			
<40%	5	0.35	0.554	Low risk group	1	15.39	0.02*
≥ 40%	13			Low-medium risk group	8		
BCL-6				High school risk group	18		
negative	7	0.53	0.465	High risk group	3		
Positive	16			C-MYC/BCL-2/BCL-6			
C-MYC/BCL-2				Non-three expression	26	0.481	0.488
Non-double expression	25	0.82	0.364	Three expression	4		
Double expression	5						

Table 6: Multivariate analysis for affecting the prognosis of patients with R/R-DLBCL.

	B	SE	Wald	P	HR	HR 95.0% CI	
						Lower limit	Upper limit
Lactate dehydrogenase	2.895	1.044	7.685	0.006	18.084	2.335	140.03

The cox stepwise regression method was used to analyze 8 variables including B symptoms, extranodal lesions, SSA, TTR, SAA+ TTR, lactate dehydrogenase, NCCN-IPI, ECOG. After regression analysis, it can be seen from the above table that lactate depletion Hydrogenase has a significant impact on the prognosis (P<0.05) and is an independent risk factor for poor prognosis.

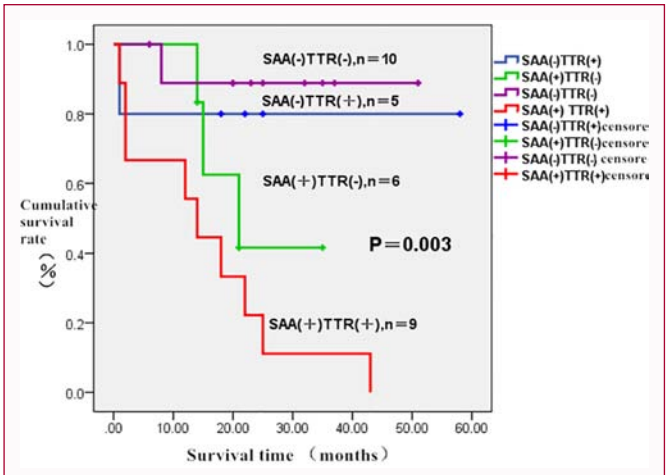

Figure 4: Survival between SAA+ TTR+ and non-SAA+ TTR+ patients in R/R-DLBCL.

disease, and staging. At the same time, each factor is further stratified in the importance of prognosis in specific extranodal locations (bone marrow, lung, brain, liver/gastrointestinal tract). NCCN-IPI was thought to consider only clinical manifestations, suggesting that R/R-DLBCL needs to be improved to achieve the best prognosis system. From the perspective of genotyping, Gene Expression Profiling (GEP) divides DLBCL into two major subtypes: Germinal Center B cell (GCB) subtype and Activated B Cell (ABC) subtype. Hans et al. [1] applied immunohistochemistry (Immunohistochemistry, IHC) basically replaces GEP; the protein level also can identify GCB and ABC. The Progression-Free Survival (PFS) and Overall Survival (OS) GCB are longer than ABC subtypes, and GCB has a better prognosis than ABC. In 2016, WHO Hematopoietic Lymphatic System Tumor Classification classified the MYC gene mutation and BCL-2/BCL-6 rearrangement in DLBCL as an independent subtype of High-Grade B-Cell Lymphoma (HGBL), known as Double-Hit Lymphoma (DHL)/Triple Fighting Lymphoma (THL) [2]. Due to the abnormally high expression of MYC and BCL-2/BCL-6 proteins, they also called Double-Expressing Lymphoma (DEL)/Triple-Expressing Lymphoma (TEL). DHL/THL, or DEL/TEL, both have the characters of rapid disease progresses and the poor prognosis [3]. From clinical manifestations, protein molecules or gene levels, there are no cheap, convenient, easy to perform, sensitive and specific markers to predict R/R-DLBCL.

In 2007, our team identified that the presence of R/R-DLBCL protein profile M/Z 11000 Da to 12000 Da was a subtype of the SAA family with or without N-terminus [4]. Subsequently, on this basis, Tris-Tricine-SDS-PAGE, shotgun-LTQ-MS protein electrophoresis, and bioinformatics technology were used to identify, and found that the serum of patients with R/R-DLBCL was highly expressed with SAA and TTR, which was significantly higher than that in remission of lymphoma, patients with chronic lymphadenitis and healthy physical examination. SAA and TTR have high value for predicting R/R-DLBCL.

SAA is a structurally highly conserved class of acute phase response proteins, a protein superfamily encoded by multiple genes. The human SAA gene is a multi-gene family containing SAA1-44 genes. It is located in the short arm of human chromosome 11. The protein expressed by the SAAl and SAA2 genes is called acute SAA (acute SAA, A-SAA), including A-SAAl protein. And A-SAA2 protein with a molecular weight of about 12,000 Da [5]. The synthesis of acute-phase protein Serum Amyloid A (SAA) is largely regulated by inflammation-related cytokines, and high concentrations of circulating SAA may be ideal markers representing acute and chronic inflammatory diseases. However, SAA can also be found in benign lesions such as sarcoidosis and pulmonary interstitial fibrosis [6]. Elevated levels of SAA can be found in the serum of patients with renal cancer metastasis [7], lung cancer [8], esophageal squamous cell carcinoma [9], breast cancer [10], and endometrial cancer [11].

The potential role of SAA in tumor pathogenesis may be due to its effect on Extracellular Matrix (ECM) adhesion proteins. SAA contains some components of ECM: Laminin and heparin/heparan sulfate binding sites [12], and has YIGSR-like and RGD-like adhesion epitopes (residues 29-42), corresponding to protein cell-binding domains on laminin and fibronectin, respectively. In addition, SAA may regulate platelet adhesion and affect tumor cell and platelet adhesion.

Tamamoto's [13] animal research reports are consistent with the conclusions of this article. Reports suggest that rfSAA can stimulate the production of MMP-9 and infiltration of feline lymphoma-derived cells, which may make SAA a new role in the progression of certain types of feline lymphoma. There have been no reports of SAA in human lymphoma occurrence and development.

The results in this study indicate that TTR is highly expressed in the serum of patients with R/R-DLBCL. TTR, also known as Pre-Albumin (PA), is a homotetramer composed of 4 identical polypeptide subunits with a molecular weight of about 54 kDa (a single subunit has a molecular weight of about 14 kDa and contains 127 amino acid residues). Studies have shown that under conditions such as malnutrition, stress, and acute infections, TTR levels will rapidly decrease. When sufficient calories are added in time, TTR levels can rise rapidly, which can be used as a sensitive indicator of the body's protein synthesis one [14].

The relationship between protein misfolding and disease is a hot spot in current molecular biology research and is the cause of many diseases. TTR mutations have been found to be associated with extensive amyloidosis in the central nervous system. However, the mechanism in TTR specifically affecting tumors remains to be studied. Many reports consistent with the results of this article that TTR expression in many tumor tissues is higher than normal tissues. For example, in lung cancer cells, the high concentration of TTR before chemotherapy is associated with treatment efficacy and prognosis [15]; in ovarian cancer, TTR is found as one of five poor

prognostic biomarkers [16]. High levels of TTR are significantly associated with poor overall survival in patients with gastric cancer metastasis [17], advanced esophageal cancer, and colorectal cancer [18,19]. However, there are few studies on TTR and lymphoma. Escher et al. [20] reported inconsistent results with this article, which may be related to the malignancy of lymphoma. This research also found that simultaneous elevation of SAA and TTR was significantly related to NCCN-IPI, which was more significant than the single factor guidance of SAA or TTR. Patients with single increase in SAA, TTR and simultaneous increase in SAA and TTR had significantly shorter survival and poor prognosis. SAA+TTR+ was associated with relapse/refractory (P=0.025, P=0.043, P=0.006), but SAA+TTR+ increased at the same time, the survival time was shorter (P<0.01). The results suggest that the combination of SAA and TTR may be used as an index for patients with pathological tissues that are not desirable. Serological markers may be a predictive index for R/R-DLBCL due to its convenience, ease of use, and easy application.

Multivariate regression analysis showed that LDH was an independent prognostic factor. LDH is a recognized serological prognostic indicator, but its specificity and sensitivity are poor, so it cannot be used as a target for targeted therapy. Although SAA and TTR are not independent prognostic factors, further research is needed to determine whether SAA and TTR can be used as candidate targets.

Combined detection of SAA and TTR had no correlation with the double/triple expression of BCL-2, C-MYC, and BCL-6 proteins, only TTR expression was correlated with C-MYC protein expression. It may because there was little sample size in this research. There were only 18 C-MYC cases, 21 BCL-2 cases, and 23 BCL-6 cases. For further studies, the sample size needs to be expanded for research, at the same time; the relationship between SAA and TTR and the double/triple strike of gene mutations are needed. Meanwhile, by quantitatively analyzing the levels of SAA and TTR, analyzing the sensitivity and specificity of diagnosing R/R-DLBCL, and determining the cutoff value, it is expected to predict relapsed/refractory cases before treatment and take better treatment measures.

Tumors have entered the era of targeted therapy, and further researches on SAA and/or TTR proteins as targets can open up a new path to reverse R/R-DLBCL, further improve the efficacy of patients and improve the prognosis of patients.

Conclusion

In this study, TTR and SAA were found to be the prognostic markers for patients with DLBCL. With the advancement of molecular technology, tumors have entered the era of targeted therapy, and targeted research on SAA and/or TTR proteins as a target can be further conducted to open up a new path for the treatment of DLBCL, which is expected to further improve the patient's efficacy and prognosis.

References

1. Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Delabie J, Ott G, et al. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood. 2004;103(1):275-82.
2. Schmitz R, Wright GW, Huang DW, Johnson CA, Phelan JD, Wang JQ, et al. Genetics and pathogenesis of diffuse large B-cell lymphoma. N Engl J Med. 2018;378(15):1396-407.
3. Swerdlow SH, Campo E, Pileri SA, Harris NL, Stein H, Siebert R, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. Blood. 2016;127(20):2375- 90.
4. Qiaohua Zhang. SELDI technology was used to detect specific protein profiles related to the prognosis of diffuse large B-cell lymphoma. Leukemia and lymphoma. 2007;16(6):419-22.
5. Uhlar CM, Whitehead AS. Serum Amyloid A, the major vertebrate acute-phase reactant. Eur J Biochem. 1999;265(2):501-23.
6. Vietri L, Bennett D, Cameli P, Bergantini L, Cillis G, Sestini P, et al. Serum Amyloid A in patients with idiopathic pulmonary fibrosis. Respir Investig. 2019;57(5):430-4.
7. Fischer K, Theil G, Hoda R, Fornara P. Serum Amyloid A: A biomarker for renal cancer. Anticancer Res. 2012;32(5):1801-4.
8. Biaoxue R, Hua L, Wenlong G, Shuanying Y. Increased serum Amyloid A as potential diagnostic marker for lung cancer: A meta-analysis based on nine studies. BMC Cancer. 2016;16(1):836.
9. Wang JY, Zheng YZ, Yang J, Lin YH, Dai SQ, Zhang G, et al. Elevated levels of serum Amyloid A indicate poor prognosis in patients with esophageal squamous cell carcinoma. BMC Cancer. 2012;12:365.
10. Zhang GJ, Sun SD, Lv H, Yang X, Kang X. Serum Amyloid A: A new potential serum correlated with the stage of breast cancer. Oncol Lett. 2012;3(4):940-4.
11. Cocco E, Bellone S, El-Sahwi K, Cargnelutti M, Buza N, Tavassoli FA, et al. Serum Amyloid A: A novel biomarker for endometrial cancer. Cancer. 2010;116(4):843-51.
12. Urieli-Shoval S, Linke RP, Matzner Y. Expression and function of serum Amyloid A, a major acute-phase protein, in normal and disease states. Curr Opin Hematol. 2000;7(1):64-9.
13. Tamamoto T, Ohno K, Gotokoshino Y, Tsujimoto H. Serum Amyloid A promotes invasion of feline mammary carcinoma cells. J Vet Med Sci. 2014;76(8):1183-8.
14. Munn DH, Mellor AL. Indoleamine 2, 3-dioxygenase and tumor-induced tolerance. J Clin Invest. 2007;117(5):1147-54.
15. Kawai H, Ota H. Low perioperative serum prealbumin predicts early recurrence after curative pulmonary resection for non-small-cell lung cancer. World J Surg. 2012;36(12):2853-7.
16. Eskander RN, Carpenter BA, Wu HG, Wolf JK. The clinical utility of an elevated-risk multivariate index assay score in ovarian cancer patients. Curr Med Res Opin. 2016;(6):1161-5.
17. Esfahani A, Makhdami N, Faramarzi E, Jafarabadi MA, Ostadrahimi A, Nahand MG, et al. Prealbumin/CRP based prognostic score, a new tool for predicting metastasis in patients with inoperable gastric cancer. Gastroenterol Res Pract. 2016;2016:4686189.
18. Kelly P, Paulin F, Lamont D, Baker L, Clearly S, Exon D, et al. Pre-treatment plasma proteomic markers associated with survival in oesophageal cancer. Br J Cancer. 2012;106(5):955-61.
19. Byström P, Berglund Å, Nygren P, Wernroth L, Johansson B, Larsson A, et al. Evaluation of predictive markers for patients with advanced colorectal cancer. Acta Oncol. 2012;51(7):849-59.
20. Escher N, Kob R, Kaatz M, Melle C, Hipler C, von Eggeling F. Confirmation of the biological significance of transthyretin as a biomarker for cutaneous T-cell lymphoma by its protein interaction partners. Mol Med Rep. 2011;4(1):157-61.