Upregulation of fatty acid synthase in MYC and BCL-2 double-expressor lymphoma

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Abstract. Diffuse large B-cell lymphoma (DLBCL) is the most common and heterogeneous lymphoid malignancy. The subtype with MYC and BCL-2 double-expressor lymphoma (DEL) was defined by its aggressive nature and poor survival outcome. Therefore, the development of effective therapies for the DEL subtype is imperative. Fatty acid synthase (FASN) activity is associated with altered lipid metabolism and aberrant protein translation in DLBCL. However, the inter-regulation of these key processes is not fully determined in DEL. In the present study, the clinical and biological impact of FASN was investigated in the DEL subtype. Initially, FASN expression levels were analyzed from a patient cohort and the data indicated that the highest FASN expression was noted in DEL tissues compared with that noted in the DLBCL and reactive lymphoid hyperplasia tissues. Patients with DEL with combined high-FASN expression indicated poorer EFS outcomes than the rest of the patients. *In vitro* data indicated that FASN was overexpressed in SU-DHL-2 and U2932 cells. Silencing FASN decreased cell growth and promoted cell apoptosis by modulating the pERK/BCL-2 signaling pathway. In conclusion, the present study indicated that FASN was overexpressed in DEL and that its expression was associated with poor survival outcomes. Furthermore, the data demonstrated that FASN regulated the biological function via the pERK/BCL-2 signaling pathway. FASN serves a critical role in the progression of DEL and its expression may be associated with the development to a more aggressive phenotype of DLBCL. Therefore, it may be considered a potential therapeutic target for DLBCL.

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Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma worldwide representing ~40% of all cases (1,2). DLBCL exhibits biological and clinical heterogeneity and ~30-40% of the patients succumb to their illness following standard first line immune-chemotherapy, including R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone) (3,4). DLBCL with rearrangement or overexpression of MYC, BCL-2 and BCL-6 is referred to as double-hit lymphoma (DHL) or double-expressor lymphoma (DEL). Due to high aggressiveness and poor prognosis, the World Health Organization recently reclassified DHL from DLBCL, not otherwise specified, or unclassified B cells between DLBCL and Burkitt lymphoma to high-grade B cell lymphoma (1). DELs, including DHL, are also characterized by an aggressive clinical course and inferior therapeutic response. In addition, DEL is relatively more common than DHL (21-34 vs. 2-10%) in newly diagnosed DLBCL and may be directly diagnosed by immunohistochemistry. Therefore, it is of considerable clinical significance to identify the underlying molecular mechanism of this disease in order to provide individualized therapy and improve patient survival (5-8).

Fatty acid synthase (FASN) is an enzyme crucial for the *de novo* synthesis of fatty acids, which acts as a metabolic driver in tumors (9-11). FASN is reported to participate in aggressive cancer phenotypes, which include the processes of uncontrolled proliferation and metastasis (12). Previous studies have revealed that FASN promotes cell growth and metastasis by activating the ERK pathway (13-17). FASN is overexpressed in DLBCL and inhibition of FASN has demonstrated potent antiproliferative and proapoptotic effects (18,19). Furthermore, FASN is reported to be associated with the development of a more aggressive phenotype and poor patient survival, including DLBCL (9,19,20). Therefore, FASN is considered a

therapeutic target in DLBCL, implying its potential function during DEL progression as well as its critical importance for the development of therapies in this subtype.

In the present study, the histopathological features of the DLBCL and DEL subtypes were investigated in association with the expression of FASN. Subsequently, the biological function of FASN and the inter-regulatory mechanisms were investigated *in vitro*. The data demonstrated that FASN-overexpression was associated with the DEL subtype and poor survival. Inhibition of FASN suppressed cell growth and promoted apoptosis via the pERK/BCL-2 signaling pathway. The present study provided evidence that FASN may be a promising therapeutic target for DEL.

Materials and methods

Patients and tissue samples. The cohort consisted of 87 patients with primary DLBCL and 15 patients with reactive lymphoid hyperplasia (RLH) who were diagnosed at the Jiangxi Cancer Hospital between March 2015 and December 2017. All cases of Epstein-Barr virus positive (EBV+) DLBCL, primary mediastinal DLBCL, primary cutaneous DLBCL, lymphomatoid granulomatosis, T-cell/histocyte rich large B-cell lymphoma, plasmablastic lymphoma and patients with a history of small mature B-cell lymphoma of various types, or insufficient follow-up period or essential clinical data were excluded. All patients were treated with R-CHOP as first line therapy. Tumor responses were assessed at the end of treatment and were classified as complete response (CR), unconfirmed complete response (CRu), partial response, stable disease or progressive disease according to the International Workshop criteria (21). Overall response rate (ORR) was defined as the proportion of patients achieving CR, CRu or PR. Ethical approval for the present study was provided by the Ethics Committee of Jiangxi Cancer Hospital. Written informed consent was obtained from all study participants.

Immunohistochemical analysis. Immunohistochemical studies were performed using formalin fixed with 10% formalin for 24 h at 25˚C, paraffin-embedded (FFPE) tissue sections, either at the time of diagnosis or for the purpose of the present study. FFPE sections were cut at a thickness of 4 μm and stained with the standard lymphoma markers mentioned below. The following antibody working solutions (0.2 mg/ml) were used for diagnosis: CD3 (cat. no. ZM-0417; Origene Technologies Inc.), CD5 (cat. no. ZM-0280; Origene Technologies Inc.), CD10 (cat. no. ZM-0283; Origene Technologies Inc.), CD20 (cat. no. kit-0001; Maxim Biotech Inc.), BCL-6 (cat. no. ZM-0011; Origene Technologies Inc.), MUM1 (cat. no. ZM-0401; Origene Technologies Inc.), Ki-67 (cat. no. ZM-0166; Origene Technologies Inc.), BCL-2 (cat. no. ZM-0010; Origene Technologies Inc.) and MYC (cat. no. ZA-0658; Origene Technologies Inc.). A primary rabbit anti-human polyclonal antibody against FASN was also used (dilution, 1:500; cat. no. ab22759; Abcam). The primary antibodies were incubated overnight at 4˚C. Protein detection was performed using a Two-step Detection kit according to the manufacturer's protocol (cat. no. PV-8000; ZSGB-BIO).

Quantitative method. The immunostaining results were evaluated by two independent pathologists separately with a light microscope (Olympus BX51) (magnification, x400). The cells of origin were determined according to the Hansalgorithm based on CD10, BCL6 and MUM1 immunostaining (22). Based on previous data that examined the optimum survival cut-offs for dichotomizing levels of expression, 40% positivity was used as a cut-off for MYC and 50% positivity for BCL-2. In the present study, DEL was defined as lymphomas demonstrating ≥40% MYC and ≥50% BCL-2 expression (23,24). FASN staining was determined by the proportion and intensity of the stained cells (11-13,25,26). FASN expression was considered positive when the stained cells were >10%. The intensity score ranged from 0 to 3 as follows: 0, no staining (<10%); 1, low staining (10-50%); 2, moderate staining (51-80%); and 3, high staining (>80%). For analytical purposes, patients with a staining score of 1-2 were categorized as low-FASN expression subjects and patients with a staining score of 3 were categorized as high-FASN expression subjects.

Cell culture. The diffused large cell lymphoma SU-DHL-2 and U2932 cell lines were obtained from the Key Laboratory of Carcinogenesis and Translational Research of Peking Universtiy Cancer Hospital & Institute. The cells were maintained in RPMI-1640 medium (Thermo Fisher Scientific, Inc.), supplemented with 15% fetal bovine serum (Hyclone; GE Healthcare Life Sciences), 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in 5% CO_2.

Western blotting. The cells were washed twice with phosphate buffered saline (PBS) and lysed in ice-cold radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) with freshly added 0.01% protease inhibitor PMSF (Amresco, LLC) and incubated on ice for 20 min. The cell lysate was centrifuged at 12,000 x g for 20 min at 4˚C. The protein concentration was determined using a Bradford assay (Bio-Rad Laboratories, Inc.). A total of 30 μg protein from each sample were run on 10% SDS-PAGE gel (TGX™ FastCast™ Acrylamide kit; Bio-Rad Laboratories, Inc.) and transferred onto a polyvinylidene difluoride membrane (EMD Millipore). The membranes were blocked with 5% BSA solution for 1 h at 25˚C and subsequently incubated with the following primary antibodies at 4˚C for overnight: Rabbit anti-human FASN (polyclonal; dilution, 1:1,000; cat. no. ab22759; Abcam), rabbit anti-human Phospho-p44/42 MAPK (Erk1/2; Thr202/Tyr204; monoclonal; dilution, 1:1,000; cat. no. 4370; Cell Signaling Technology, Inc.), rabbit anti-human MYC (Y69; monoclonal; dilution, 1:1,000; cat. no. ab32072; Abcam), rabbit anti-human BCL-2 antibody (E17; monoclonal; dilution, 1:1,000; cat. no. ab32124; Abcam) and rabbit anti-human β-actin (polyclonal; dilution, 1:5,000; cat. no. ab8227; Abcam). A horseradish peroxidase-conjugated goat anti-rabbit IgG H&L secondary antibody (1:10,000, ab205718, Abcam) was incubated at 25˚C for 1 h. Protein detection was performed using an enhanced chemiluminescence kit (ECL; Thermo Fisher Scientific, Inc.) with an ECL Imager (Thermo Fisher Scientific, Inc.).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher

Scientific, Inc.). Reverse transcriptase reactions were performed using cDNA and M-MLV reverse transcriptase (Promega Corporation). The RT reaction conditions were as follows: 65˚C for 2 min, 42˚C for 1 h, 70˚C for 10 min and 4˚C for 2 min. For mRNA detection, FASN and GAPDH mRNA expression were analyzed using SYBR-Green qPCR reagents according to the manufacturer's protocols (Applied Biosystems; Thermo Fisher Scientific, Inc.). Gene specific primers (10 μM) were used at a final concentration of 0.2 μM with cDNA (1 μl) as template. The cycling conditions were as follows: 95˚C for 2 min, followed by 39 cycles of 95˚C for 15 sec and 60˚C for 60 sec. The sequences of the primers are listed in Table I and were verified by Primer-BLAST (NCBI). The data were analyzed using the $2^{-\Delta\Delta Cq}$ relative quantification method (27).

Cell Counting Kit-8 (CCK-8) cytotoxicity assay. The proliferation of the cells was measured using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.). SU-DHL-2 and U2932 cell suspensions were seeded onto 96-well plates at a density of 2,000 cells/well and cultured at 37˚C in 5% CO_2. A total of 20 μl CCK-8 solution was added to each well and the plates were incubated for 4 h in a 37˚C incubator at the indicated time points (1, 2 and 3 days). The optical density levels were measured by a microplate reader at 450 nm according to the manufacturer's protocols.

Survival rate assay. The same number of cells ($1.0x10^6$ cells/well) was cultured in the 24-well plates for 1, 2 and 3 days. Cell viability was quantified by adding 100 μl of 0.4% trypan blue dye to 100 μl of cell suspension at 25˚C for 5 min and counting the numbers of stained (non-viable) and unstained (viable) cells on a haemocytometer.

Transfection of FASN silencing RNA. For transfection of small interfering (siRNA) targeting FASN (FASN silencer; cat. no. s5031; Thermo Fisher Scientific Inc.) and non-targeting control siRNA (Silencer® Select Negative Control No. 1 siRNA; cat. no. 4390843; Thermo Fisher Scientific Inc.). The sequences of siRNA targeting FASN were as follows: Forward, 5'-CCGUGGACCUGAUCAUCAATT-3', and reverse, 5'-UUGAUGAUCAGGUCCACGGCG-3'. HiPerfect (Qiagen, Inc.) transfection reagent was added to the medium without serum in order to yield a final HiPerFect concentration of 0.5% (v/v). siFASN/negative control (NC) was added to the aforementioned medium, mixed (final concentration 5-10 nM), incubated for 10 min at room temperature and finally added to the cells. Subsequently, cells were cultured at 37˚C with 5% CO_2 for 48 h and collected for subsequent experiments.

Flow cytometry. The two cell lines were transfected with silencing RNA targeting FASN/NC (siFASN/NC) for 2 days. The FITC Annexin V Apoptosis Detection kit with propidium iodide (cat. no. 640914; BioLegend, Inc.) was used to detect the cell apoptotic rate. Flow cytometry data were collected with a BD LSR Fortessa cell analyzer and analyzed using FlowJo Software (FlowJo LLC; Version 10).

Statistical analysis. Event-free survival (EFS) was defined as the time between diagnosis or disease progression and the time of relapse or death from any cause or to the participant termination date (31 Dec, 2019). Patients without an event were censored at the time point of last known follow-up. Survival curves were estimated using the Kaplan-Meier curve and comparisons were made using the log-rank test. Univariate analyses were made using the chi-square test and the log-rank test. The data are presented as the mean ± standard error of the mean. A two-tailed Student's t-test was used to compare differences between the two groups. Statistical analysis was performed using the SPSS version 15.0 (SPSS, Inc.) and the graphs were generated by the GraphPad Prism version 5.0 (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Sequences of all primers.

Primer name	Sequences (5'-3')
GAPDH (homo sapiens) forward	ATTGTCAGCAATGCATCCTG
GAPDH (homo sapiens) reverse	ATGGACTGTGGTCATGAGCC
FASN (homo sapiens) forward	CAACTCACGCTCCGGAAA
FASN (homo sapiens) reverse	TGTGGATGCTGTCAAGGG

FASN, fatty acid synthase.

Results

Clinicohistopathological features of DEL and non-DEL patients. A total of 87 eligible cases of *de novo* DLBCL were included in the present study. All patients were immunocompetent. The demographic and clinicopathological data are presented in Table II. MYC and BCL-2 DEL was identified in 36 (41%) cases. Additionally, 44 cases (51%) were MYC-positive and 43 cases (49%) were MYC-negative. A total of 71 cases (82%) were BCL-2-positive and 16 cases (18%) were BCL-2-negative. The medium age at diagnosis was 57 and 53 years in the DEL and non-DEL groups, respectively. Age and sex distributions were similar in the two groups. DEL was significantly associated with late stage (Ann Arbor stage III/IV), increased lactate dehydrogenase levels, extra nodal sites >2, PS score >1 and IPI score ≥3. The Hans algorithm was used and the findings indicated that the non-GCB subtype was more prevalent in the DEL than in the non-DEL groups (89 vs. 65%; P=0.012).

FASN expression in DLBCL and DEL patients. Immunohistochemical analyses were performed to determine the expression levels of FASN in 87 DLBCL tissues and 15 RLH tissues. FASN expression was considered positive when the stained cells were >10%. The tissues were divided into the three groups according to FASN expression scores as follows: Negative, low-FASN expression, high-FASN expression (Fig. 1A). The results revealed that FASN protein was located in the cytoplasm and germinal center.

Table II. Comparison of clinicopathological features between non-DEL and DEL subgroup in 87 patients with diffuse large B-cell lymphoma.

Characteristic	Total n=87 (%)	Non-DEL n=51 (%)	DEL n=36 (%)	P-value
Median age (range)	55 (20-84)	53 (24-84)	57 (20-81)	
Age >60 years	30 (34)	16 (31)	14 (39)	0.499
Male:female	46:41	25:26	21:15	0.531
Ann Arbor stage III/IV	42 (42)	18 (35)	24 (67)	0.004[a]
Elevated LDH	47 (48)	19 (37)	28 (78)	0.000[a]
ENS >2	20 (23)	6 (12)	14 (39)	0.004[a]
PS score >1	25 (29)	9 (18)	17 (47)	0.004[a]
IPI score ≥3	19 (22)	6 (12)	14 (39)	0.004[a]
BM involvement$^+$	8 (9)	2 (4)	6 (17)	0.061
CNS involvement$^+$	5 (6)	2 (4)	3 (8)	0.645
B symptom	9 (10)	3 (6)	6 (17)	0.154
GCB:non-GCB subtype	22:65	18:33	4:32	0.012[a]
Ki67 ≥80%	27 (31)	15 (29)	12 (33)	0.851
ORR	66 (76)	46 (90)	20 (56)	0.000[a]

[a]Pearson's Chi-Square Test. DEL, double-expressor lymphoma; LDH, lactate dehydrogenase; ENS, extra nodal sites; PS score, performance status score; IPI score, international prognostic index score; BM$^+$, bone marrow involved; CNS$^+$, central nervous system involved; GCB, germinal center B-cell; ORR, objective response rate.

FASN expression was positive in all DLBCL samples and high-FASN expression was observed in 72% of DLBCL samples. Furthermore, the data indicated that FASN expression was significantly higher in DLBCL compared with that in RLH tissues (2.72±0.05 vs. 1.07±0.18; P<0.0001; Fig. 1B). In DLBCL tissues, the expression of FASN was higher in the DEL group than in the non-DEL group (2.86±0.06 vs. 2.57±0.08; P=0.008; Fig. 1C). The clinicohistopathological characteristics were analyzed according to FASN expression (Table III). High FASN expression was significantly associated with MYC and BCL-2 combined expression (P=0.027). In addition, high expression of FASN was associated with MYC-positive cases (P=0.017) and those with a high Ki67 index (P=0.043).

Survival analysis. All patients in the cohort received immunochemotherapy [rituximab plus cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP)] as the first line therapy. The data showed that DEL patients exhibited an inferior ORR (56 vs. 90%) and survival (11 months vs. not reached), compared with those noted in the non-DEL group. Subsequently, the prognostic impact of FASN in DLBCL and DEL patients was investigated. High FASN expression indicated poorer ORR than the low-expression group (71 vs. 88%; P=0.092; Table III). The 2-year EFS rate in the high FASN expression group was 59% (95% CI: 52-65%), while the rate in the low expression group was 83% (95% CI: 81-91). However, the log-rank test indicated no significant differences with regard to these parameters (P=0.069; Fig. 2A). With a median follow up of 20 months, the median EFS of the two groups had not been reached by the termination of follow up. Subsequently, survival analysis was performed on DEL patients with combined high FASN expression. These patients indicated significantly poorer EFS rates and times than the remaining patients (2-year EFS rate, 27 vs. 88%, P<0.0001; median EFS, 9.5 months vs. not arrived; Fig. 2B). Ten factors that had significant differences from univariate analysis were included and analyzed, and COX regression analysis was performed. The results demonstrated that increased LDH and double expression of MYC and BCL-2 (DEL) are independent factors that influenced the prognosis and survival in all the patients (Table SI).

Silencing of FASN inhibits cell growth and promotes apoptosis of DLBCL cells. The two DLBCL cell lines, SU-DHL-2 and U2932, which have been previously reported as non-GCB type and MYC/BCL-2 amplification-type, respectively, were cultured *in vitro* (28,29). The experimental data indicated that SU-DHL-2 and U2932 cells exhibited high expression levels of FASN, as well as MYC and BCL-2 protein amplification (Fig. 3A). To further investigate the function of FASN, its silencing was achieved by siRNA targeting in order to obtain FASN-silenced cells (Fig. 3B and C). CCK-8 assay revealed that inhibition of FASN attenuated cell growth (Fig. 3D). The survival assay indicated that deletion of FASN decreased the cell survival rate (Fig. 3E). The flow cytometry assay indicated that silencing of FASN significantly accelerated the cell apoptotic death (Fig. 3F and G), which was accompanied by cleavage and activation of the apoptosis effector caspase-3 (Fig. S1). These results confirmed that FASN was required for the aggressive phenotype noted in DLBCL.

Silencing of FASN suppresses the pERK1/2/Bcl-2 signaling pathway in the U2932 cell line. The molecular pathway of FASN was investigated in combination with the induction of apoptosis in U2932 cells. Previous studies have demonstrated that

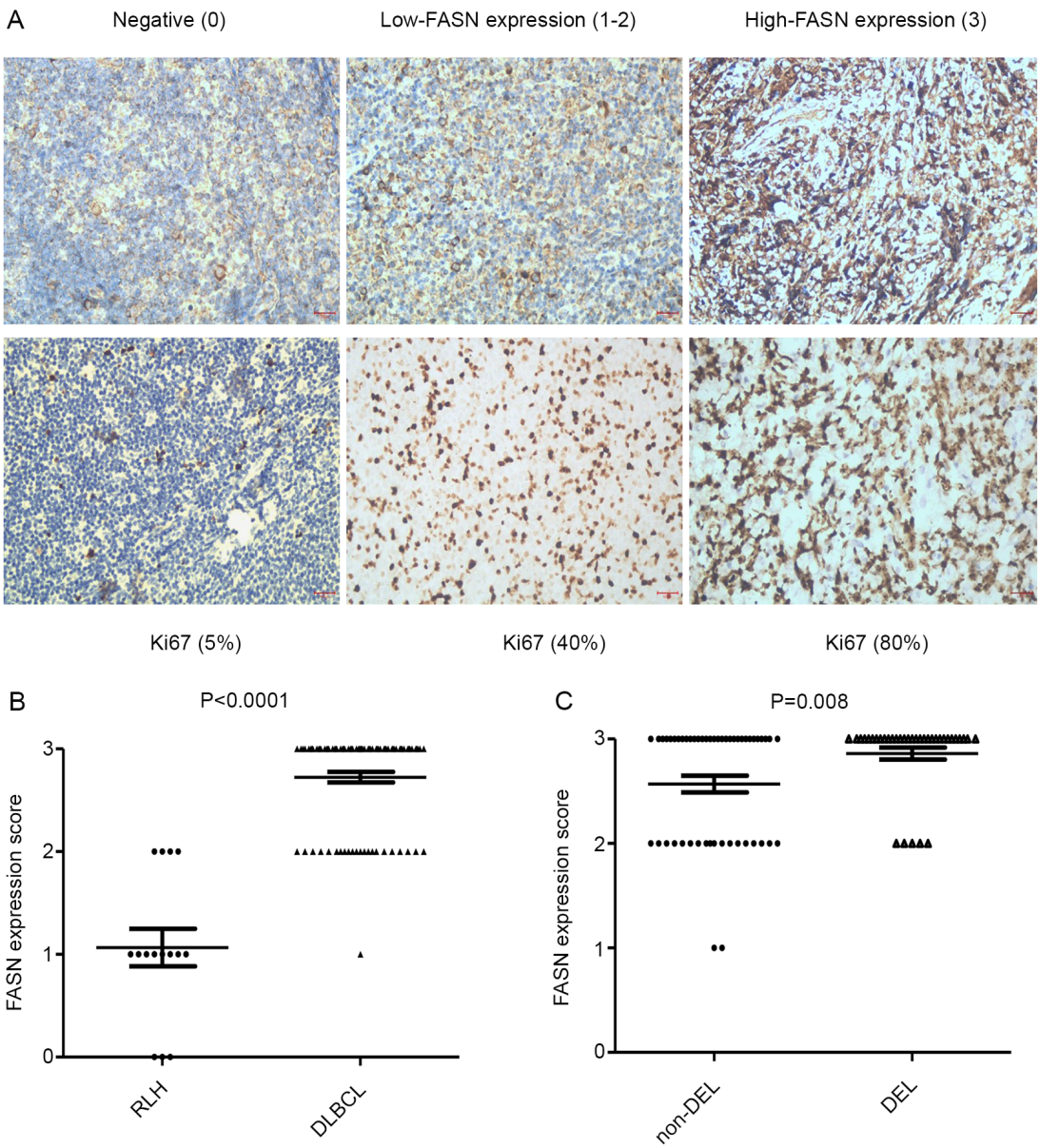

Figure 1. Upregulation of FASN protein in DEL patients. (A) Representative immunohistochemical staining of FASN and Ki67 proteins in tissues (magnification, x400). Tissues were divided into three groups according to FASN expression intensity scores: 0, negative (left), 1-2, low-FASN expression (middle), 3, high-FASN expression (right). (B) The FASN expression scores in 87 patients with DLBCL, compared with 15 patients with RLH. (C) The FASN expression score in DEL, compared with non-DEL patients. Values shown are the mean ± standard error of the mean. Scale bar, 100 μm. FASN, fatty acid synthase; DLBCL, diffuse large B-cell lymphoma; RLH, reactive lymphoid hyperplasia; DEL, double-expressor lymphoma.

FASN may regulate ERK1/2 phosphorylation and the expression of the anti-apoptotic proteins, including BCL-xl (14,15). Therefore, additional experiments were performed to determine whether FASN exerts it functions by regulating the pERK1/2-associated signaling pathway in DLBCL. Silencing of FASN decreased the levels of p-ERK1/2 and BCL-2, while total ERK1/2 levels remained unchanged, indicating that it may suppress ERK1/2 phosphorylation and further decrease BCL-2 expression. Nevertheless, the expression of MYC was not significantly altered (Fig. 4).

Discussion

DEL associated with co-expression of MYC and BCL-2 is recognized as a poor prognostic subgroup of DLBCL. The present study assessed the clinicopathological features of 87 patients with DLBCL who were recruited between March 2015 and December 2017 in Jiangxi Cancer Hospital. MYC and BCL-2 DEL cases were identified in 41% of all cases. DEL patients exhibited a decreased ORR (56 vs. 90%) and survival time (11 months vs. not arrived), compared with that of non-DEL patients. These findings were mostly consistent with the results of previous studies (23,24,30-32). Treatment of DEL is mainly restricted to R-CHOP treatment. Despite several efforts, the therapeutic efficacy of current targeted agents with the exception of rituximab remains controversial (33-36). Therefore, particular focus has been given to the identification of novel molecular targets for this specific subtype.

FASN is considered an oncogene in carcinogenesis and has emerged as a potential therapeutic target for lymphoma (37,38).

Table III. Clinicohistopathological characteristics according to FASN expression in 87 patients with diffuse large B-cell lymphoma.

Characteristic	Total n=87 (%)	FASN Low expression n=24 (%)	FASN High expression n=63 (%)	P-value
Median (range)	55 (20-84)	55 (23-81)	55 (20-84)	
Age >60 years	30 (34)	11 (46)	19 (30)	0.210
Male:female	46:41	13:11	33:30	1.000
Ann Arbor stage	42 (48)	12 (50)	30 (48)	1.000
Elevated LDH	47 (54)	10 (42)	37 (59)	0.229
ENS >2	20 (23)	2 (8)	18 (29)	0.050
PS score >1	26 (30)	7 (29)	19 (30)	1.000
IPI score ≥3	20 (23)	4 (17)	16 (25)	0.570
BM involvement+	8 (9)	2 (8)	6 (10)	0.691
CNS involvement+	5 (6)	0 (0)	5 (8)	0.316
B symptom	9 (10)	2 (8)	7 (11)	1.000
GCB:non-GCB subtype	22:65	1:2	2:7	0.287
Ki67 ≥80%	28 (32)	4 (17)	26 (42)	0.043[a]
MYC +	44 (51)	7 (29)	37 (59)	0.017[a]
BCL-2 +	71 (82)	19 (79)	52 (83)	0.761
MYC+BCL-2+(DEL)	36 (41)	5 (21)	31 (49)	0.027[a]
ORR	66 (76)	21 (88)	45 (71)	0.092

[a]Pearson's Chi-Square Test. FASN, fatty acid synthase. DLBCL, diffuse large B-cell lymphoma; DEL, double-expressor lymphoma; LDH, lactate dehydrogenase; ENS, extra nodal sites; PS score, performance status score; IPI score, international prognostic index score; BM+, bone marrow involved; CNS+, central nervous system involved; GCB, germinal center B-cell; ORR, objective response rate.

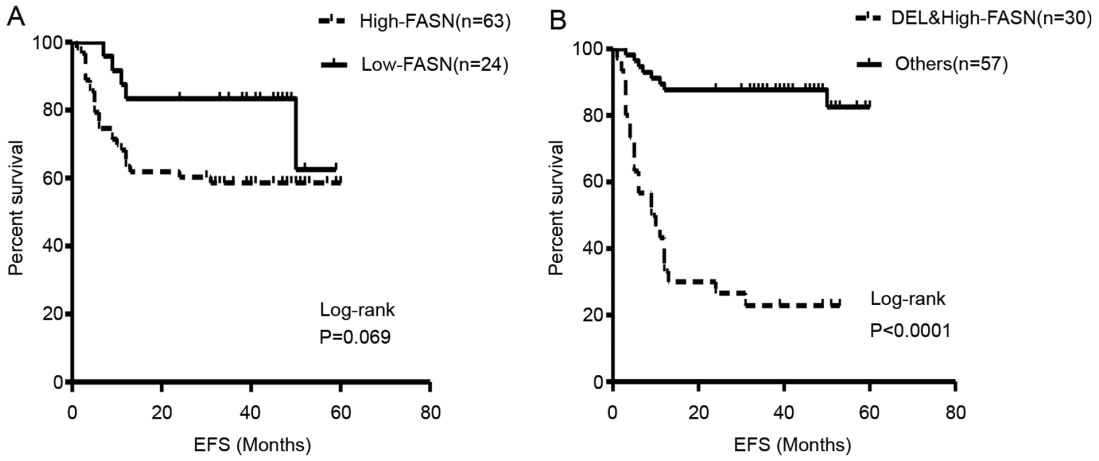

Figure 2. EFS analysis of DLBCL patients with different subtypes following frontline R-CHOP treatment. (A) Comparison of EFS between high-FASN expression and low-FASN expression patients. (B) Comparison of EFS between DEL patients with high-FASN expression and the other patients. EFS, event-free survival; DLBCL, diffuse large B-cell lymphoma; DEL, double-expressor lymphoma; FASN, fatty acid synthase.

In the present study, substantial evidence is presented regarding the association of FASN expression with DLBCL. To begin with, the data indicated that FASN was overexpressed in DLBCL, compared with RLH samples, while it was present in highly proliferative tissues characterized by high Ki-67 index (P=0.043). Furthermore, FASN protein distribution was different between the DLBCL and RLH tissues. In RLH tissues, FASN expression was restricted in the germinal center B-cells, confirming the association between FASN and BCL-2. However, FASN expression was not restricted in the germinal center and was widely distributed in the DLBCL tissues. In addition, FASN expression was significantly higher in DEL than non-DEL tissues. FASN was reported to be associated with cancer progression (14-16). The present findings provided evidence of an active role of FASN in DLBCL progression to a more aggressive phenotype (DEL).

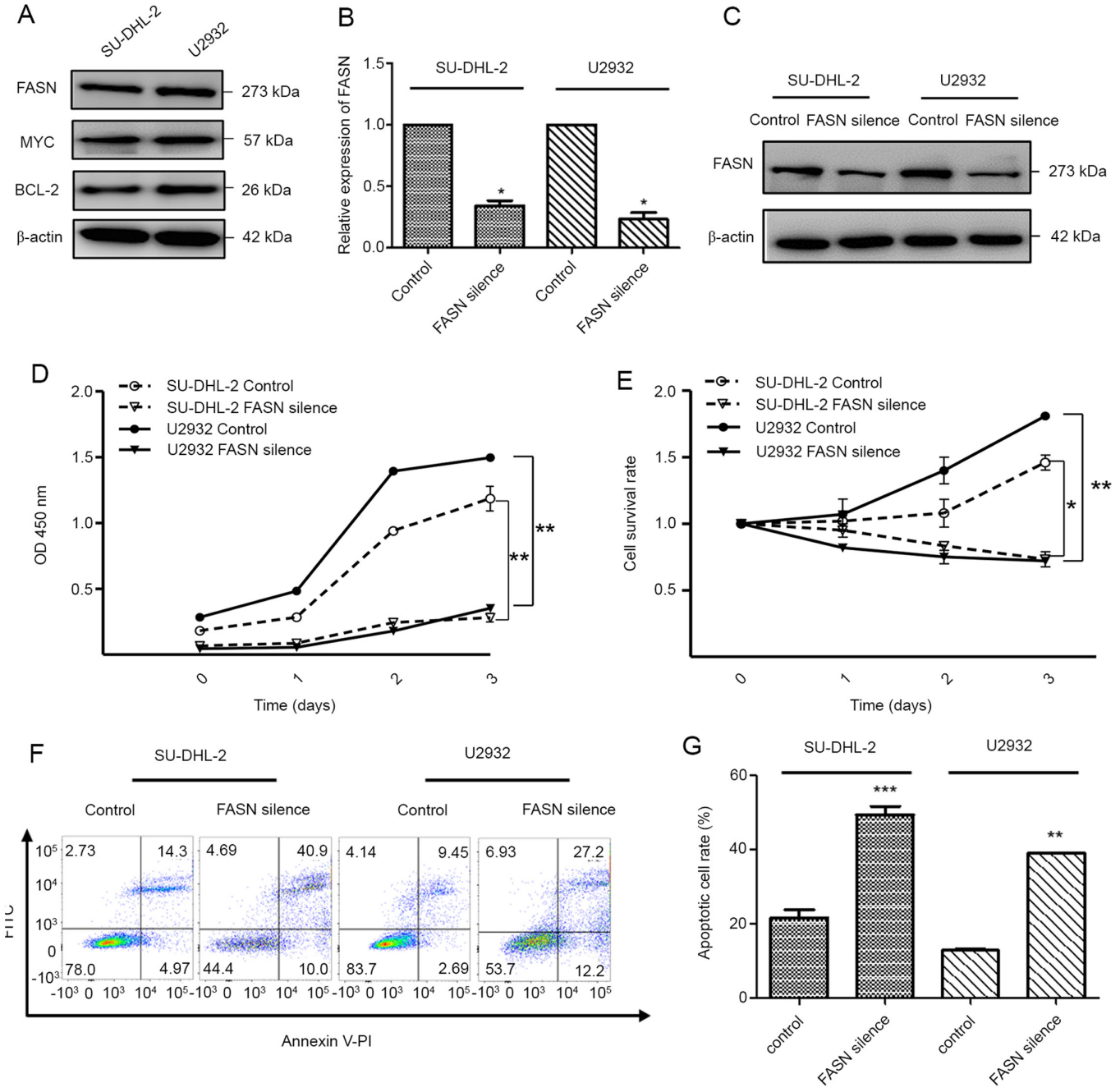

Figure 3. Silencing of FASN inhibited cell growth and promoted apoptosis of diffuse large B-cell lymphoma cells. (A) Expression of FASN, MYC and BCL-2 protein in SU-DHL-2 and U2932 cells. FASN expression in cells treated with siFASN/Control for 2 days was detected by (B) reverse transcription-quantitative polymerase chain reaction and (C) western blotting. (D) Cell Counting Kit-8 assay was performed to detect the proliferation abilities following transfection with siFASN/Control for 0, 1, 2 and 3 days. (E) Cell survival rate of cells following transfection with siFASN/Control for 0, 1, 2 and 3 days. (F) Apoptotic indexes and (G) rate of cells were detected by fluorescence-activated cell sorting-based Annexin V-FITC/PI double staining following transfection with siFASN/Control for 2 days. Values shown are the mean ± standard error of the mean. *P<0.05, **P<0.01, ***P<0.001. FASN, fatty acid synthase; si, small interfering RNA.

Survival analysis demonstrated that DEL patients with high-FASN expression exhibited poorer EFS, compared with that noted in other patients (2-year EFS rate, 27 vs. 88%, P<0.0001; median EFS, 9.5 months vs. not reached). However, following the analysis it was not possible to demonstrate a significant and direct association between FASN and EFS. This finding may be associated with the retrospective nature of the present study and the relatively small number of the patients involved. The findings suggested that high-FASN expression patients revealed lower ORR (71 vs. 88%, P=0.092) and 2-year EFS (59 vs. 83%, P=0.065), compared with that of low-FASN expression patients. Future studies should focus on a larger number of uniformly-treated patients that may aid the clarification of the prognostic and predictive value of FASN in DEL.

A previous study suggested that FASN inhibitors may trigger apoptosis and suppress the expression of the c-Met kinase in DLBCL (19). The present study aimed to elucidate the biological function of FASN and the associated molecular mechanisms *in vitro*. MYC/BCL-2 amplification has been reported in SU-DHL-2 and U2932 cell lines and as a result these two cell lines were used as DEL cells. The results

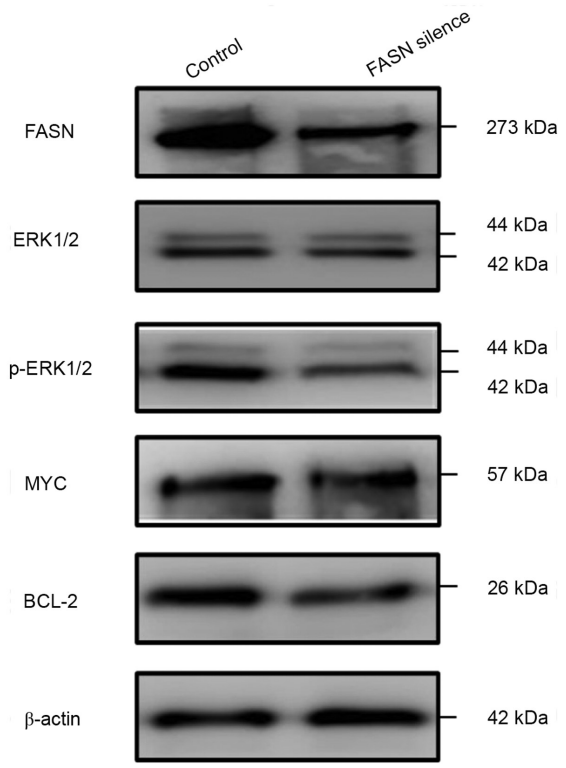

Figure 4. Silencing of FASN suppressed the pERK1/2/BCL-2 signaling pathway in the U2932 cell line. Expression of FASN, EKR1/2, p-ERK1/2 and BCL-2 protein in U2932 cells following transfection with siFASN/Control for 2 days. β-actin was used as a loading control. FASN, fatty acid synthase; si, small interfering RNA.

indicated that FASN was highly expressed in SU-DHL-2 and U2932 cells and that its knockdown decreased tumor growth and promoted apoptosis. These results confirmed that FASN promoted cell growth and apoptosis resistance in DEL, as well as in DLBCL (19,37). The FASN/ERK pathway is a vital signaling pathway in cancer (14-15). The present study indicated that silencing FASN decreased p-ERK and BCL-2 expression, suggesting that FASN may regulate cell growth and apoptosis via the p-ERK/BCL-2 signaling pathway in DEL. However, MYC expression levels remained unaltered. This finding may be attributed to the fact that MYC is regulated by several genes, whereas FASN is not its direct upstream target.

In addition, there are certain limitations to the present study. The biological function of FASN and the exact mechanism linking p-ERK/BCL-2 signaling to FASN expression requires further confirmation by ectopic expression of FASN in the negative control cell lines. In the following gain-functional assay, two cell lines with low expression of FASN should be added to confirm the function with ectopic expression of FASN. The length of FASN mRNA is ~8.4 kb and its full-length cloning is difficult. The establishment of FASN overexpression models is a difficult task and our previous studies used FASN cDNA ORF (NCBI Ref Seq BC007909) to upregulate FASN, suggesting that this method is possible. Therefore, the gain/loss of function experiments on FASN are designed to be implemented in future studies. It has been highlighted that FASN-overexpression in tumor tissues is associated with the serum levels of FASN (26,39,40). In the present study, the association between the clinical prognostic factors of DEL and FASN expression levels (tumor and serum) was investigated.

The present study highlighted that deregulation of FASN may be associated with the pathogenesis of DEL and provided a rational preclinical setting for the therapeutic applications of FASN inhibitors in DEL. Future clinical studies should focus on the assessment of the effectiveness of FASN inhibitors in combination with R-CHOP as therapeutic agents for the treatment of DEL. Therefore, FASN may serve as a potential therapeutic target in DEL patients.

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Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

XZ, YS and JGL designed the study. XZ obtained funding, purchased the reagents and materials, and provided other support. XZ, ZL and QL acquired the data. XZ and WZ analyzed and interpreted the data, XZ drafted the manuscript. XZ, WZ, JGL and YS critically revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Jiangxi Cancer Hospital and was performed in accordance with the Declaration of Helsinki and the guidelines of the Ethics Committee of Jiangxi Cancer Hospital. Written informed consent was obtained from all patients for the use of their clinical tissues.

Patient consent for publication

Written informed consent was obtained from all patients for publication.

Competing interests

The authors declare that they have no competing interests.

References

1. Cazzola M: Introduction to a review series: The 2016 revision of the WHO classification of tumors of hematopoietic and lymphoid tissues. Blood 127: 2361-2364, 2016.
2. Lagunas-Rangel FA: A new history: The 2016 revision of the WHO classification of tumors of hematopoietic and lymphoid tissues. Austin Leuk 1: 1001, 2016.

3. Lukenbill J and Hill B: Relapsed/Refractory diffuse large B-cell lymphoma: Review of the management of transplant-eligible patients. Leuk Lymphoma 56: 293-300, 2015.
4. Jain P, Fayad LE, Rosenwald A, Young KH and O'Brien S: Recent advances in de novo CD5$^+$ diffuse large B cell lymphoma. Am J Hematol 88: 798-802, 2013.
5. Reagan PM and Davies A: Current treatment of double hit and double expressor lymphoma. Hematology Am Soc Hematol Educ Program 2017: 295-297, 2017.
6. Saumyaranjan M, Arun P, Moien L, Ansh G, Parashant R, Minakshi S, Ajay G, Ranjit S and Atul S: Clinicopathological profile of primary double expressor lymphoma. Clin Lymphoma Myeloma Leuk 19 (Suppl 1): S257, 2019.
7. Choe JY, Park M, Yun JY, Na HY, Go H, Kim HJ, Oh S and Kim JE: PELI1 expression is correlated with MYC and BCL6 expression and associated with poor prognosis in diffuse large B-cell lymphoma. Mod Pathol 29: 1313-1323, 2016.
8. Smith SM: Aggressive B-cell lymphoma: The double-hit and double-expressor phenotypes. Clin Adv Hematol Oncol 15: 40-42, 2017.
9. Danilova OV, Dumont LJ, Levy NB, Lansigan F, Kinlaw WB, Danilov AV and Kaur P: FASN and CD36 predict survival in rituximab-treated diffuse large B-cell lymphoma. J Hematop 6: 11-18, 2013.
10. Menendez JA and Lupu R: Fatty acid synthase (FASN) as a therapeutic target in breast cancer. Expert OpinTher Targets 21: 1001-1016, 2017.
11. Wang H, Xi Q and Wu G: Fatty acid synthase regulates invasion and metastasis of colorectal cancer via wnt signaling pathway. Cancer Med 5: 1599-1606, 2016.
12. Menendez JA and Lupu R: Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. Nat Rev Cancer 7: 763-777, 2007.
13. Liu ZL, Wang G, Peng AF, Luo QF, Zhou Y and Huang SH: Fatty acid synthase expression in osteosarcoma and its correlation with pulmonary metastasis. Oncol Lett 4: 878-882, 2012.
14. Sun TH, Zhong X, Song HH, Liu JM, Li JG, Leung F, Lu WW and Liu ZL: Anoikis resistant mediated by FASN promoted growth and metastasis of osteosarcoma. Cell Death Dis 10: 298, 2019.
15. Hu N, Li Y, Zhao Y, Wang Q, You Jc, Zhang Xd and Ye Lh: A novel positivefeedbackloopinvolvingFASN/p-ERK1/2/5-LOX/LTB4/FASN sustains high growth of breast cancer cells. Acta Pharmacol Sin 32: 921-929, 2011.
16. Bian Y, Yu Y, Wang S and Li L: Up-regulation of fatty acid synthase induced by EGFR/ERK activation promotes tumor growth in pancreatic cancer. Biochem Biophys Res Commun 463: 612-617, 2015.
17. Akiyama T, Choong PF and Dass CR: RANK-Fc inhibits malignancy via inhibiting ERK activation and evoking caspase-3-mediated anoikis in human osteosarcoma cells. Clin Exp Metastasis 27: 207-215, 2010.
18. Kapadia B, Nanaji NM, Bhalla K, Bhandary B, Lapidus R, Beheshti A, Evens AM and Gartenhaus RB: Fatty acid synthase induced S6Kinase facilitates USP11-eIF4B complex formation for sustained oncogenic translation in DLBCL. Nat Commun 9: 829, 2018.
19. Uddin S, Hussain AR, Ahmed M, Bu R, Ahmed SO, Ajarim D, Al-Dayel F, Bavi P and Al-Kuraya KS: Inhibition of fatty acid synthase suppresses c-met receptor kinase and induces apoptosis in diffuse large B-cell lymphoma. Mol Cancer Ther 9: 1244-1255, 2010.
20. Gonzalez-Guerrico A and Lupu R: Fatty acid synthase (FASN) as a therapeutic target for breast cancer metastasis. Cancer Res 69 (Suppl 24): 6160, 2009.
21. Cheson BD, Fisher RI, Barrington SF, Cavalli F, Schwartz LH, Zucca E and Lister TA: Recommendations for initial evaluation, staging, and response assessment of hodgkin and non-hodgkin lymphoma: The lugano classification. J Clin Oncol 32: 3059-3068, 2014.
22. Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Delabie J, Ott G, Müller-Hermelink HK, Campo E, Braziel RM, Jaffe ES, *et al*: Conformation of molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. Blood 103: 275-282, 2004.
23. Johnson NA, Slack GW, Savage KJ, Connors JM, Ben-Neriah S, Rogic S, Scott DW, Tan KL, Steidl C, Sehn LH, *et al*: Concurrent expression of MYC and BCL2 in diffuse large B-cell lymphoma treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone. J Clin Oncol 30: 3452-3459, 2012.
24. Bogusz AM, Kovach AE, Le LP, Feng D, Baxter RH and Sohani AR: Diffuse large B-cell lymphoma with concurrent high MYC and BCL2 expression shows evidence of active B-cell receptor signaling by quantitative immunofluorescence. PLoS One 12: e0172364, 2017.
25. Giró-Perafita A, Sarrats A, Pérez-Bueno F, Oliveras G, Buxó M, Brunet J, Viñas G and Miquel TP: Fatty acid synthase expression and its association with clinico-histopathological features in triple-negative breast cancer. Oncotarget 8: 74391-74405, 2017.
26. Puig T, Blancafort A, Casoliva G, Oliveras G, Casas M, Buxo M, Saiz E, Viñas G, Dorca J and Porta R: Prospective analysis of fatty acid synthase (FASN) in breast cancer tissue of early-stage breast cancer patients. Cancer Res 72 (24 Suppl): P4-09-10, 2012.
27. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta DeltaC(T)) method. Methods 25: 402-408, 2001.
28. Yao S, Xu F, Chen Y, Ge Y, Zhang F, Huang H, Li L, Lin D, Luo X, Xu J, *et al*: Fbw7 regulates apoptosis in activated B-cell like diffuse large B-cell lymphoma by targeting Stat3 for ubiquitylation and degradation. J Exp Clin Cancer Res 36: 10, 2017.
29. Tarantelli C, Gaudio E, Kwee I, Cascione L, Bernasconi E, Hillmann P, Stathis A, Stussi G, Fabbro D, Wicki A, *et al*: The dual PI3K/mTOR inhibitor PQR309 has synergistic activity with other targeted agents in diffuse large B cell lymphomas. Blood 126: 4005, 2015.
30. Takahashi H, Miura K, Nakagawa M, Sugitani M, Amano Y, Kurita D, Sakagami M, Ohtake S, Uchino Y, Kodaira H, *et al*: Negative impact of concurrent overexpression of MYC and BCL2 in patients with advanced diffuse large B-cell lymphoma treated with dose-intensified immunochemotherapy. Leuk Lymphoma 57: 2784-2790, 2016.
31. Li L, Zhang X, Zhang T, Song Z, Hu G, Li W, Li L, Qiu L, Qian Z, Zhou S, *et al*: Prognostic significance of BCL-2 and BCL-6 expression in MYC-positive DLBCL. Clin Lymphoma Myeloma Leuk 18: e381-e389, 2018.
32. Ma Z, Niu J, Cao Y, Pang X, Cui W, Zhang W and Li X: Clinical significance of 'double-hit' and 'double-expression' lymphomas. J Clin Pathol 73: 126-138, 2020.
33. Johnston PB, LaPlant B, McPhail E, Habermann TM, Inwards DJ, Micallef IN, Colgan JP, Nowakowski GS, Ansell SM and Witzig TE: Everolimus combined with R-CHOP-21 for new, untreated, diffuse large B-cell lymphoma (NCCTG 1085 [Alliance]): Safety and efficacy results of a phase 1 and feasibility trial. Lancet Haematol 3: e309-e316, 2016.
34. Chiappella A, Witzig TE, Vitolo U, Gascoyne R, Russo J, Amoroso B, Hudak K, Ogunkanmi A, Xu Y, Ruiz W, *et al*: ROBUST: Phase III randomized study of LENALIDOMIDE/R-CHOP VS PLACEBO/R-chop in untreated ABC-type diffuse large B-cell lymphoma and feasibility of cell of origin subtyping. Hematol Oncol 35: 419-419, 2017.
35. Brower V: Ibrutinib promising in subtype of DLBCL. Lancet Oncol 16: e428, 2015.
36. Westin JR, Nastoupil L, Hagemeister F, Fayad L, Young K, McDonnell T, Chuang H, Ahmed S, Nair R, Steiner R, *et al*: SMART start: Rituximab, lenalidomide, and ibrutinib alone prior to combination with chemotherapy for patients with newly diagnosed diffuse large B-cell lymphoma. Hematol Oncol 37: 78-79, 2019.
37. Gelebart P, Zak Z, Anand M, Belch A and Lai R: Blockade of fatty acid synthase triggers significant apoptosis in mantle cell lymphoma. PLoS One 7: e33738, 2012.
38. Kant S, Kumar A and Singh SM: Myelopoietic efficacy of orlistat in murine hosts bearing T cell lymphoma: Implication in macrophage differentiation and activation. PLoS One 8: e82396, 2013.
39. Long QQ, Yi Yx, Qiu J, Xu CJ and Huang PL: Fatty acid synthase (FASN) levels in serum of colorectal cancer patients: Correlation with clinical outcomes. Tumour Biol 35: 3855-3859, 2014.
40. Ezzeddini R, Taghikhani M, Somi MH, Samadi N and Rasaee MJ: Clinical importance of FASN in relation to HIF-1α and SREBP-1c in gastric adenocarcinoma. Life Sci 224: 169-176, 2019.