

Original Article

CYFIP1 is a potential tumor suppressor in human diffuse large B-cell lymphoma

Xi Liu¹, Keman Xu², Jing Wang¹, Yanfang Wang¹, Lin Fu¹, Xiaoyan Ke¹

¹Department of Hematology and Lymphoma Research Center, Peking University Third Hospital, Beijing, China;

²Northeastern University, Boston, MA, USA

Received February 26, 2017; Accepted April 26, 2017; Epub June 1, 2017; Published June 15, 2017

Abstract: CYFIP1 was reported to be deleted in common human epithelial cancers, which suggested its role as a putative tumor suppressor. However, the role of CYFIP1 in human diffuse large B-cell lymphoma (DLBCL) remains ill-defined. Herein, we aimed to investigate the expression and function of CYFIP1 in DLBCL. We detected the expression of CYFIP1 in 48 tumor specimens of human DLBCL by immunohistochemistry. Negative CYFIP1 expression was seen in 85.4% (41/48) of human DLBCL tissues. However, no significant correlations were found between CYFIP1 expression and clinic-pathological features of DLBCL including gender, age, histological type, and the expression of Ki-67, Bcl-2, Bcl-6, and mum-1. Reduced CYFIP1 expression was verified in two DLBCL cell lines (DB and SU-DHL-4) as compared to normal human peripheral blood lymphocytes using quantitative RT-PCR and western blotting. The biological functions of CYFIP1 in DLBCL were determined by cell viability, cell apoptosis and cell cycle. The overexpression of CYFIP1 suppressed cell growth and induced apoptosis, while had no influence on cell cycle in DLBCL cells. We employed western blotting assay to explore the molecules regulated by CYFIP1 overexpression and found that the activation of Ras/Raf/ERK signaling was suppressed by CYFIP1 in DLBCL. CYFIP1 is therefore down-regulated and functions as a potential tumor suppressor and clinical biomarker in human DLBCL.

Keywords: CYFIP1, human DLBCL, tumor suppressor

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin's lymphoma (NHL) in adults with an annual incidence of more than 25,000 cases. Furthermore, NHL accounts for approximately 30-40% of newly diagnosed global cases of lymphoma [1, 2]. Current standard therapy for patients with DLBCL includes rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) and approximately 60% of patients experience long-term remission using this regimen [3]. However, despite improvements in therapy, DLBCL is still associated with a high mortality rate [4], wherein approximately one-third of patients present with DLBCL will be refractory to therapy or relapse from the disease [5]. Therefore, understanding the molecular mechanisms of DLBCL pathogenesis and discovering new biomarkers could assist in the development of early detection approaches and targeted therapies for this disease.

CYFIP1 (cytoplasmic FMRP-interacting protein 1) is a cytoplasmic protein and was first identified as a protein that interacts with Fragile X mental retardation protein (FMRP) [6]. Silva et al. suggested that CYFIP1 was a putative tumor suppressor that regulated invasive behavior. The study found that CYFIP1 was deleted in human epithelial cancers. Moreover, reduced expression of CYFIP1 was observed during invasion of tumor, which was associated with a poor prognosis in the same tumor types [7]. In addition, Silva et al. also proposed that CYFIP1 could influence tumorigenesis, and did so by affecting cytoskeletal dynamics, cell-cell and cell-substratum adhesion. Moreover, Silva et al. showed that CYFIP1 knock-down cooperated with activated Ras to promote tumor progression [7]. Another study found that decreased expression of CYFIP1 was significantly associated with the occurrence and lymph node metastasis of acute lymphoblastic leukemia (ALL), and proposed that CYFIP1 was a tumor suppressor that was important in the occurrence and development of ALL [8].

CYFIP1 is a putative tumor suppressor

However, relatively little is known about the direct impact of CYFIP1 on tumor progression in DLBCL. Thus, our current study was aimed at exploring whether CYFIP1 played a role as a tumor suppressor in DLBCL, and as previously reported [7, 8]. To comprehensively study the role of CYFIP1 in DLBCL, we first detected the expression of CYFIP1 in human DLBCL tissues, and compared the differences in expression in DLBCL cell-lines with normal human peripheral blood lymphocytes. Secondly, functional studies related to cell proliferation, cell apoptosis and cell cycle in DB and SU-DHL-4 cells were performed to reveal the functional significance of CYFIP1 in DLBCL. Finally, human cancer pathway assay was used to explore molecular mechanisms of CYFIP1 in DLBCL.

Materials and methods

Clinical data and samples

Paraffin-embedded tissue samples from 48 DLBCL patients diagnosed between 2008 and 2015 at Peking University Third Hospital (Beijing, China) were studied. All patients were diagnosed by hematoxylin and eosin (H&E) histochemical staining and the immunophenotype was defined according to the World Health Organization Classification system [9], with complete clinico-pathological analyses and follow-up data. All patients received combination regimens including CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisolone) and CHOP-like regimens. Some patients received additional radiotherapy, surgical therapy, and hematopoietic stem cell transplantation. Normal peripheral blood lymphocytes of four candidates involved in our study were obtained from the Clinical Laboratory of Peking University Third Hospital. Informed consent was obtained from all patients and volunteer candidates. This work was approved by local ethics committees of Peking University Third Hospital.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue samples were used for further immunohistochemical analyses. Classification of DLBCL into germinal center (GC) and non-GC subgroups was based on immunohistochemical analysis of the relative expression of CD10, Bcl6 and melanoma-associated antigen (mutated)-1 (mum-1) according to the algorithm described

by Hans et al. [10]. CYFIP1, CD10, Bcl-2, Bcl-6 and mum-1 were immunostained using the diaminobenzidine (DAB) histochemistry kit (Molecular Probes, Invitrogen, CA, USA) according to the manufacturer's instructions on formalin-fixed and paraffin-embedded tissues as previously reported [11]. Sections were incubated overnight at 4°C with primary antibodies, including anti-CYFIP1 (Abcam, Cambridge, MA, USA), anti-CD10, anti-Bcl-2, anti-Bcl-6, anti-mum-1 (Novocastra, UK), followed by incubation with the appropriate biotinylated secondary antibody and horseradish peroxidase (HRP)-conjugated avidin. Finally, DAB staining on the sections was visualized by bright field light microscopy. For CYFIP1 expression, cases were considered negative if fewer than 10 percent of tumor cells showed positive staining as previously reported [7]. Ki-67 expression was considered negative if the median percentage of cells with nuclear expression of Ki-67 was less than 80% in our study. For others, cases were considered positive if 30 percent of tumor cells showed positive staining [12].

Cell culture and reagents

Human DLBCL cell-lines DB and SU-DHL-4 were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). All cells were cultured in RPMI-1640 (Gibco, Grand Island, NY, USA), supplemented with 10% FBS (HyClone, South Logan, UT, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were routinely cultured at 37°C in a humidified incubator containing 5% CO₂. The cells in logarithmic growth phase were used for experiment.

Plasmid construction, lentivirus production and transduction

The human *cyfip1* (Gene ID: 23191) targeting sequence and a negative non-targeted control sequence were used to generate recombinant lentiviral particles. These recombinant lentiviruses were prepared and tittered to a density of 1×10⁸ TU/ml (transfection units), and the multiplicity of infection (MOI) was 15 (DB) and 30 (SU-DHL-4) respectively. Antibiotic-resistant clones were isolated and maintained in medium containing 20 µg/ml puromycin (Sigma-Aldrich, St. Louis, MO, USA). Overexpression of CYFIP1 was confirmed by quantitative RT-PCR and western blotting analysis. The infected

CYFIP1 is a putative tumor suppressor

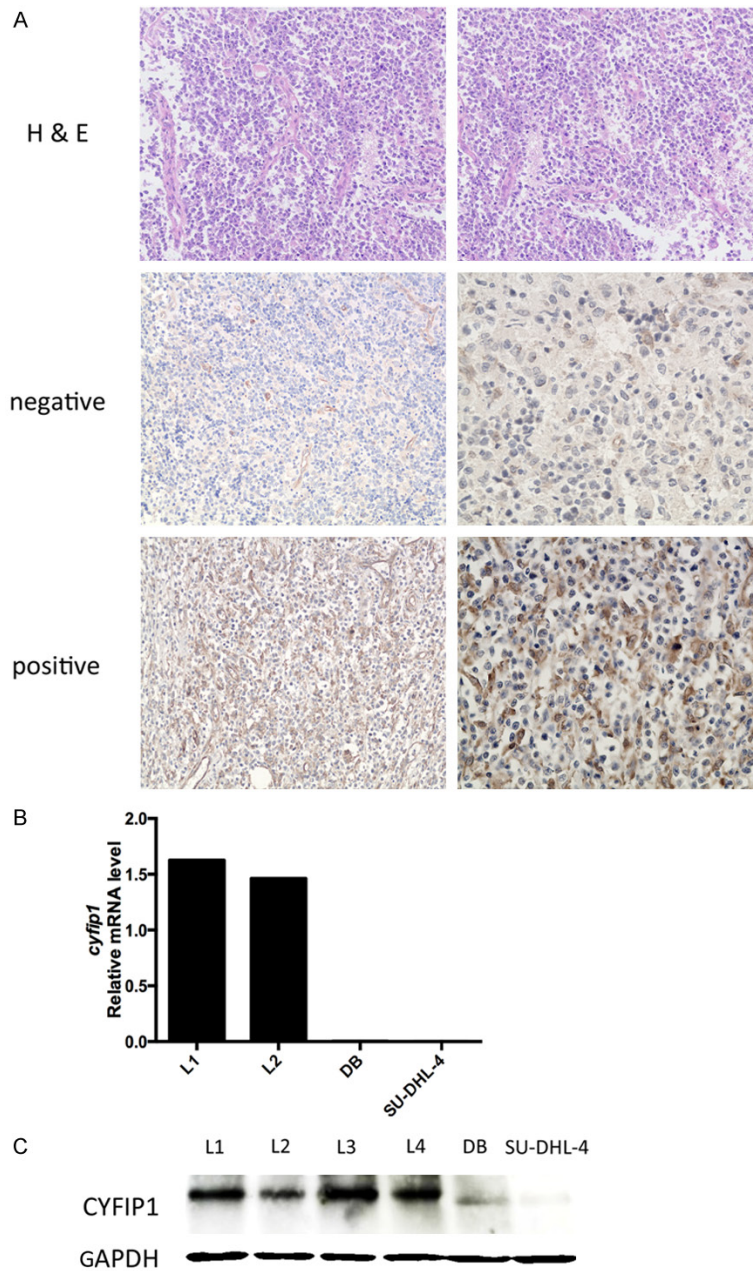


Figure 1. CYFIP1 was down-regulated in human diffuse large B-cell lymphoma (DLBCL) tissues and cell-lines. A: CYFIP1 expression in DLBCL tissues as determined by immunohistochemistry. The microscopic features of DLBCL in H&E-stained tumor sections (upper panel) and representative cases of negative and positive CYFIP1 expression (middle and lower panel) were shown. The images show cytoplasmic CYFIP1 staining. B: The mRNA levels of CYFIP1 in DB, SU-DHL-4 cells and normal human peripheral blood lymphocytes of two candidates (L1, and 2) as determined by quantitative RT-PCR. C: Protein levels of CYFIP1 in DB, SU-DHL-4 cells and normal human peripheral blood lymphocytes of four candidates (L1 to 4) as determined by western blotting.

cells comprised CYFIP1 overexpression (CYFIP1) and negative non-targeted control groups (CON). Both groups were used for the following experiments.

Reverse-transcription and quantitative RT-PCR

Total RNA was extracted from all above mentioned DLBCL cell-lines and human peripheral blood lymphocytes using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and then reverse transcribed for complementary DNA (cDNA) using a Reverse Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Braunschweig, Germany). The primers used for PCR were as follows:

5'-GTACGGCTC TCCTGGTATCC-3' (forward), 5'-CTCTTTCACATGGACTCGCG-3' (reverse) for *cyfip1* and 5'-TGA AGG TCG GAGTCA ACG GAT TTG GT-3' (forward), 5'-CAT GTGGGC CAT GAG GTC CAC CAC-3' (reverse) for GAPDH. The PCR conditions were as follows: 95°C for 2 min, then 25-30 cycles (*cyfip1*: 30 cycles; GAPDH: 25 cycles) at 95°C for 30 sec, 55-60°C (*cyfip1*: 60°C; GAPDH: 55°C) for 30 sec, 72°C for 30 sec, and finally 72°C for 2 min. The GAPDH gene was applied as an internal control and calculated to obtain the adjusted cycle threshold (Δ CT) value for the expression levels of the *cyfip1* gene. The adjusted CT value was calculated according to the relative expression of *cyfip1* = $2^{-\Delta$ CT}. Δ CT = [CT value *cyfip1*]-[CT value of GAPDH]. The specific method for calculating relative expression levels of the tumor suppressor gene was referenced from previously published literature [13].

Western blotting

The cells were lysed on ice and total proteins were extracted following standard procedures. The concentrations of protein were determined by BCA protein assay kit (Pierce, Rockford, IL, USA). Proteins were separated by 12% sodium

CYFIP1 is a putative tumor suppressor

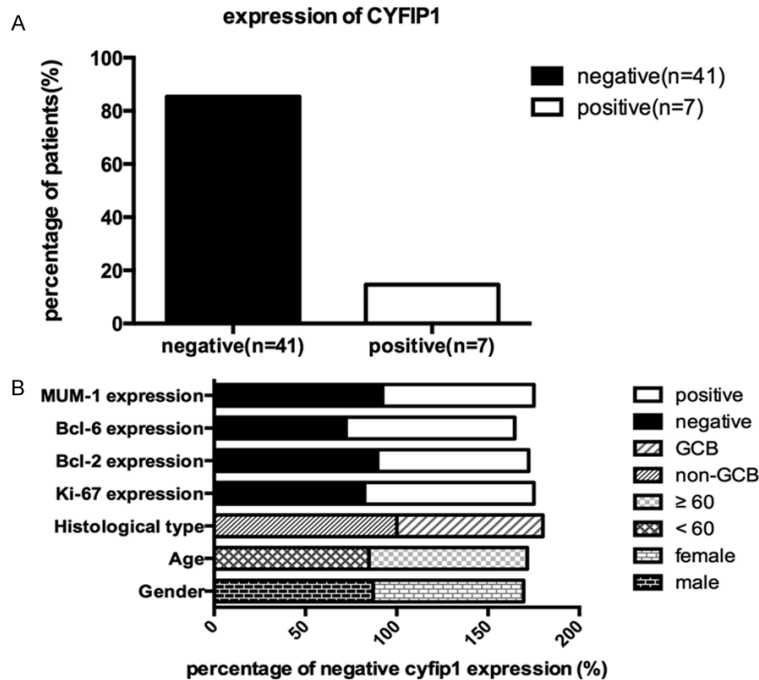


Figure 2. Correlation between CYFIP1 expression and clinic-pathological features. A: Of the 48 DLBCL samples observed by immunohistochemistry, 85.4% (41/48) of the samples showed negative staining, while 14.6% (7/48) showed positive CYFIP1 expression as compared to the corresponding adjacent normal tissues. B: No significant correlations were found between CYFIP1 expression and the selected clinic-pathological features including gender, age (< 60.0 years vs. \geq 60.0 years), histological type (GCB vs. non-GCB) and the expression of Ki-67, Bcl-2, Bcl-6, and mum-1 ($P > 0.05$).

dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Hybond; GE Healthcare, Bucks, UK). The membranes were blocked in 5.0% bovine serum albumin (BSA) for 1 h at room temperature. After blocking, the membranes were sequentially incubated with primary antibodies, including anti-CYFIP1 (Abcam, Cambridge, MA, USA), anti-PCNA, anti-Caspase-3, anti-Ras, anti-Raf, anti-ERK and anti-phospho-ERK (CST, Beverly, MA, USA), anti-GAPDH (Multisciences, Shanghai, China), and then with HRP-conjugated anti-rabbit or mouse secondary antibodies (eBioscience, San Diego, CA, USA). Signals were detected with the LAS500 device (GE, New York City, NY, USA).

Cell viability assay

The Cell Counting Kit-8 (CCK-8) (Dojindo, Tokyo, Japan) was used to study cell viability according to the manufacturer's instructions. In brief, a cell suspension was inoculated into

96-well plates at a density of 3.0×10^3 cells/well and incubated in serum-free medium for 0, 24, 48 and 72 h. At every time point, 10 μ l CCK-8 was added to each well and the plate was further incubated for 4 h in the dark at 37°C and 5% CO₂. The absorbance values were measured at a wavelength of 450 nm and final values were calculated. Each assay was performed three times.

Cell apoptosis assay

The Annexin V-PE/7-AAD Apoptosis Detection Kit (Yeasen, Shanghai, China) was used to detect apoptotic cells. Following the manufacturer's instructions, the cells (1×10^6 /sample) were harvested and washed twice with pre-cooled PBS. Next, the cells were resuspended in 100 μ l binding buffer with 5 μ l aliquots of Annexin V/PE and 10 μ l aliquots of 7-AAD. The cells were then exposed to the mixed

solution for 15 min in the dark at room temperature. The cell samples were assessed by multi-parameter flow cytometry within 1 h. Samples were analyzed on a BD FACS Calibur (BD Bioscience, San Jose, CA, USA). Each experiment was performed three times.

Cell cycle analysis

Cells were seeded into a six-well plate and harvested after being transfected and incubated for 48 h. The cells (1×10^6 /sample) were washed twice with pre-cooled PBS and fixed in 70% ethanol overnight at -20°C, and then resuspended in 1.0 mL PBS with 100 μ g/ml RNase A (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 30 min. After being stained with propidium iodide (PI; 500 μ g/ml) (Sigma-Aldrich, St. Louis, MO, USA) for 10 min at room temperature, the cells were collected on a BD FACS Calibur (BD Bioscience, San Jose, CA, USA), and the data analyzed using the ModFit LT software program (Verity Software House, Topsham, ME, USA).

CYFIP1 is a putative tumor suppressor

Table 1. Characteristic of 48 cases of diffuse large B-cell lymphoma

Characteristic	Case no.	CYFIP1 expression		P value
		Negative	Positive	
Gender				
Male	31	27	4	P = 0.686
Female	17	14	3	
Age				
< 60	33	28	5	P = 0.622
≥ 60	15	13	2	
Histological type				
Non-GCB	13	13	0	P = 0.166
GCB	35	28	7	
Ki-67 expression				
Negative	34	28	6	P = 0.656
Positive	14	13	1	
BCL-2 expression				
Negative	19	17	2	P = 0.687
Positive	29	24	5	
BCL-6 expression				
Negative	22	17	5	P = 0.223
Positive	26	24	2	
MUM-1 expression				
Negative	13	12	1	P = 0.656
Positive	35	29	6	

Statistical analysis

The statistical analysis of data sets was performed using the SPSS version 23.0 software program (IBM Corporation, Armonk, NY, USA). Data are shown as the mean standard deviation of triplicate values for each experiment. Statistical comparisons were performed using analysis of variance (ANOVA). The relationship between CYFIP1 expression and various clinic-pathological parameters was analyzed using Fisher's Exact Test for categorical variables. An alpha value of $P < 0.05$ was considered statistically significant.

Results

Correlation between CYFIP1 expression and clinic-pathological characteristics

CYFIP1 expression was evaluated in DLBCL tissues and their corresponding adjacent normal tissues in 48 DLBCL patients. As determined by immunohistochemistry (IHC), CYFIP1 expression was significantly down-regulated in 85.4% (41/48) of DLBCL tissues as compared with the

corresponding adjacent normal tissues (**Figures 1A** and **2A**), while only 14.6% (7/48) of DLBCL tissues showed positive CYFIP1 expression. (**Figures 1A** and **2A**). Positive CYFIP1 expression was predominantly located in the cytoplasm of vascular endothelial cells; however, the expression was significantly decreased in tumor tissues (**Figure 1A**). The correlations between negative CYFIP1 expression and the various clinic-pathological features of the 48 DLBCL cases are presented in **Table 1**.

Among 48 patients with DLBCL, 31 were male (64.6%), which was concordant with previously reported percentage of male patients (63%) [14]. The mean age of the patients was 49.7 years, and the median age was 48.0 years. No significant correlations were found between CYFIP1 expression and the listed clinic-pathological features, which included gender, age (< 60.0 vs. ≥ 60.0 years), histological type and the expression of Ki-67, Bcl-2, Bcl-6, and mum-1 ($P > 0.05$, **Table 1**; **Figure 2B**). Of the seven patients that positively expressed CYFIP1, the histological type was GCB and six patients failed to express Ki-67 (**Table 1**).

CYFIP1 is down-regulated in DLBCL cell-lines

To further validate CYFIP1 expression at the transcriptional and translational levels, we performed quantitative RT-PCR and western blotting in two DLBCL cell-lines, including DB and SU-DHL-4 cells, and normal human peripheral blood lymphocytes. We observed that the mRNA levels of CYFIP1 were notably repressed in both DLBCL cell-lines as compared with peripheral blood lymphocytes (**Figure 1B**). Western blotting analysis confirmed the reduction of CYFIP1 expression in DLBCL cells (**Figure 1C**). These concordant data clearly showed CYFIP1 expression was restrained in DLBCL.

CYFIP1 overexpression inhibits cell proliferation

To better understand the role of CYFIP1 in the progression of DLBCL, DB and SU-DHL-4 with low and steady levels of endogenous CYFIP1 (**Figure 1B** and **1C**) were transfected with *cyfip1* or empty vector via lentiviruses. Enhanced CYFIP1 expression was evidenced by quantitative RT-PCR and western blotting (**Figure 3A** and **3B**).

CYFIP1 is a putative tumor suppressor

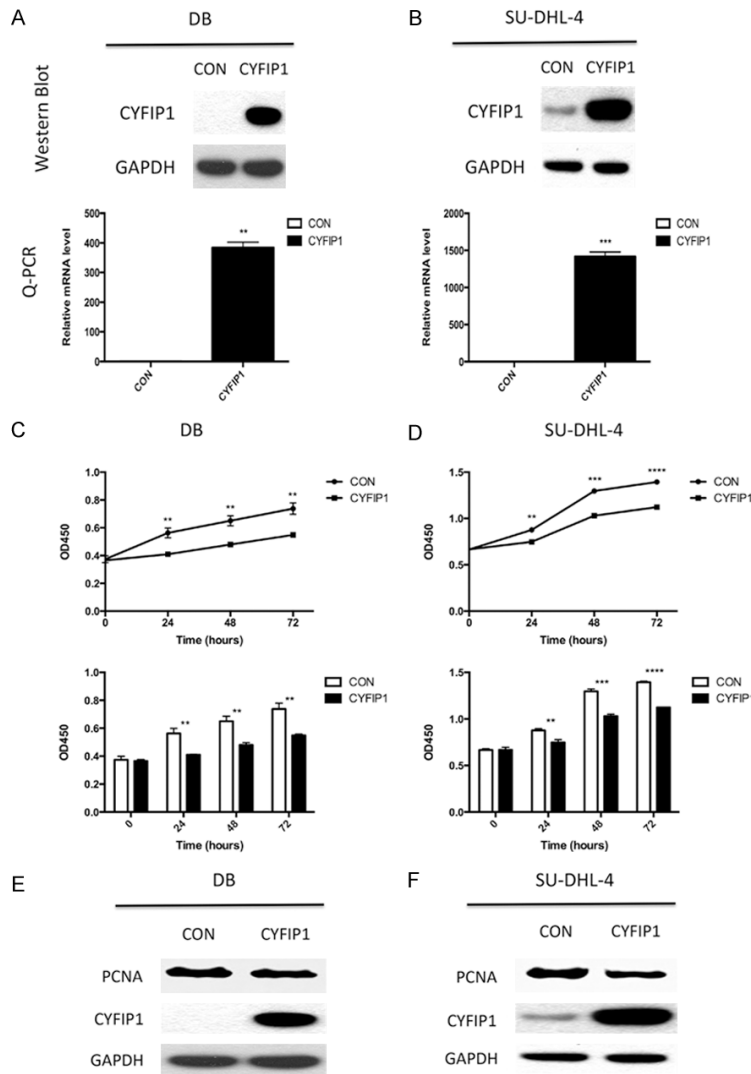


Figure 3. CYFIP1 overexpression inhibited cell growth in DB and SU-DHL-4 cells. A and B: CYFIP1 overexpression in DB and SU-DHL-4 cells was performed using lentivirus transduction. The mRNA and protein levels of CYFIP1 in stable cell-lines were detected by quantitative RT-PCR and western blotting. GAPDH was used as a loading control. C and D: DB/CYFIP1, SU-DHL-4/CYFIP1 and control cells were seeded into 96-well plates, following which, CCK-8 assay was performed to quantify cell growth. Compared to the relative control groups (CON), the cell proliferation of DB/CYFIP1 and SU-DHL-4/CYFIP1 cells decreased at 24, 48 and 72 h, respectively ($P = 0.0017$, $P = 0.0018$, $P = 0.0015$ vs. $P = 0.0031$, $P = 0.0001$, $P < 0.0001$). Each assay was performed three times. E and F: PCNA levels were detected in CYFIP1 over-expressing and control cells by western blotting. GAPDH was used as a protein loading control.

CCK-8 assay was performed to evaluate the effects of CYFIP1 overexpression on tumor cell proliferation. The stable overexpression of CYFIP1 in DB and SU-DHL-4 cells significantly reduced cell growth. The cell proliferation of DB/CYFIP1 and SU-DHL-4/CYFIP1 cells was decreased after 24, 48 and 72 h respectively,

compared to the relative control groups ($P = 0.0017$, 0.0018 , and 0.0015 vs. $P = 0.0031$, 0.0001 , and < 0.0001 ; **Figure 3C** and **3D**). Furthermore, we assessed markers reflecting tumor cell proliferation ability by western blotting assay. The expressions of PCNA in DB/CYFIP1 and SU-DHL-4/CYFIP1 cells were significantly decreased compared to the control groups (**Figure 3E** and **3F**). Decreases in PCNA expression further confirmed the inhibitory effect of CYFIP1 on cell proliferation.

CYFIP1 overexpression induces apoptosis and minimally influences tumor cell cycle

To determine whether the observed growth suppression by CYFIP1 was due to apoptosis induction, CYFIP1 over-expressing cells and control cells were stained with Annexin V/7-AAD and analyzed by flow cytometry. As compared to the control groups, the apoptosis rates of DB/CYFIP1 and SU-DHL-4/CYFIP1 cells were increased by 51.0% and 21.3% respectively after 48 h of incubation ($P = 0.0104$ vs. $P < 0.001$; **Figure 4A** and **4B**). We then performed western blotting to assess the expression of the apoptosis marker caspase-3. Consistent with an increased induction of apoptosis, the levels of cleaved caspase-3 in both DB/CYFIP1 and SU-DHL-4/CYFIP1 cells were enhanced. (**Figure 4C**

and **4D**) The results indicated that CYFIP1 over-expression promoted cell apoptosis.

To further explore the mechanism by which CYFIP1 inhibited cell growth, we then explored the effect of CYFIP1 on cell cycle distribution. Our data showed that the cell cycle progression

CYFIP1 is a putative tumor suppressor

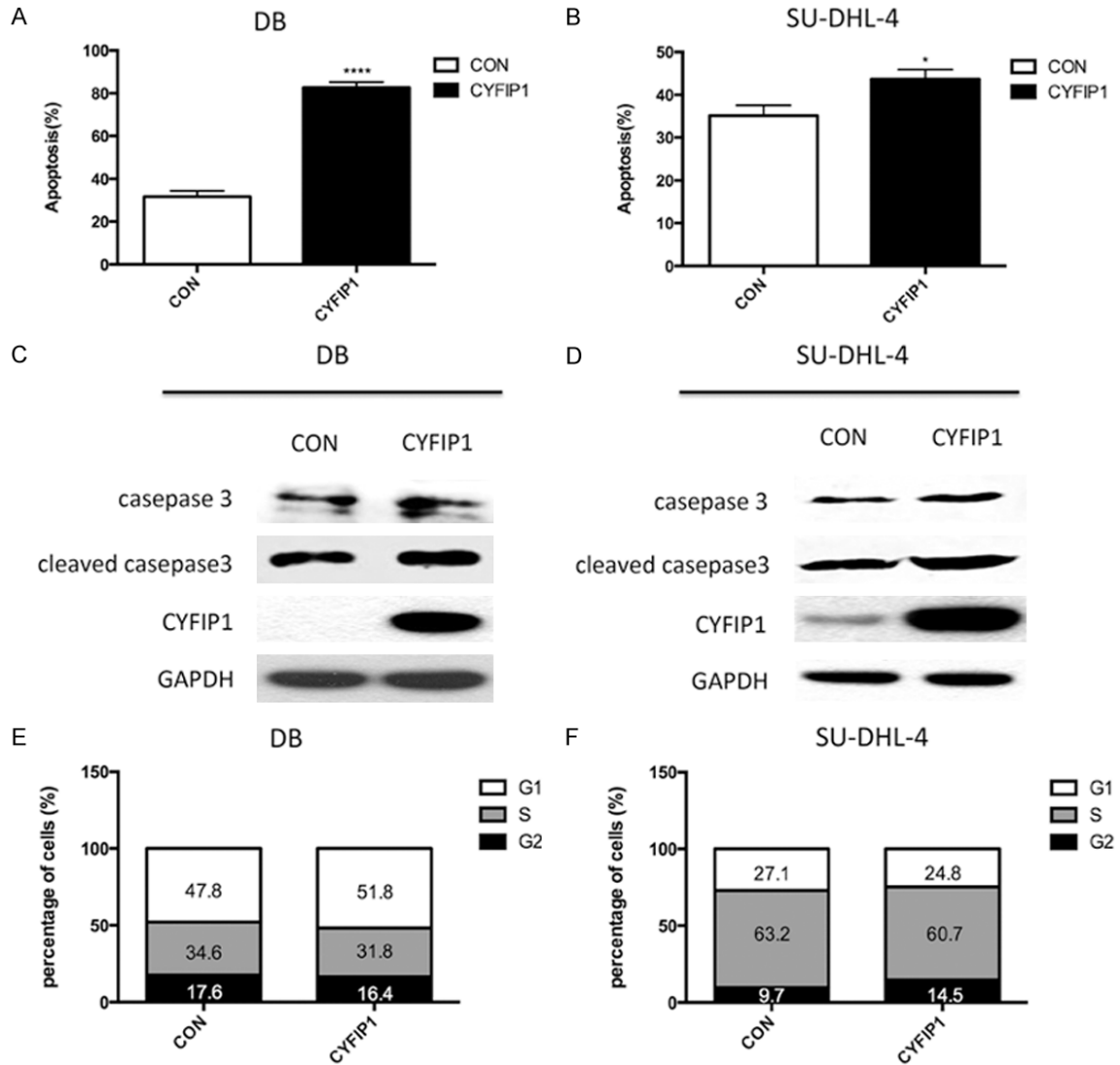


Figure 4. CYFIP1 overexpression promoted apoptosis and minimally influence cell cycle. A and B: DB/CYFIP1, SU-DHL-4/CYFIP1 and the corresponding control cells were seeded into six-well plates. The cells were stained with Annexin-V/7-AAD and analyzed by flow cytometry after incubation for 48 hours. The apoptosis rates of DB/CYFIP1 and SU-DHL-4/CYFIP1 cells increased by 51.0% and 21.3% as compared to the relative controls (CON) respectively ($P = 0.0104$ vs. $P < 0.001$). C and D: The levels of caspase-3 and cleaved caspase-3 were tested in CYFIP1 overexpressing and control cells using western blotting. GAPDH was used as a protein loading control. E and F: Cell cycle progression was analyzed by flow cytometry after DB/CYFIP1, SU-DHL-4/CYFIP1 and control cells were incubated for 48 h. The proportion of cells in G1, S and G2 phases of the cell cycle was shown. Each assay was performed three times.

in DB and SU-DHL-4 cells was not inhibited by overexpression of CYFIP1 (**Figure 4E and 4F**). The proportion of cells in S-phase of the cell cycle was somewhat dampened by 2.8% and 2.5% respectively in the CYFIP1 group compared with the control group of both DB and SU-DHL-4 cells ($P > 0.05$). This indicated that CYFIP1 overexpression inhibits cell proliferation without influencing cell cycle.

CYFIP1 overexpression suppressed Ras/Raf/ERK signaling

In follow-up experiments, we carried out a series of western blotting analysis to understand the mechanism by which CYFIP1 might give rise to the observed inhibitory effects on cell proliferation. Total protein was extracted from stable cells and relative control cells in

CYFIP1 is a putative tumor suppressor

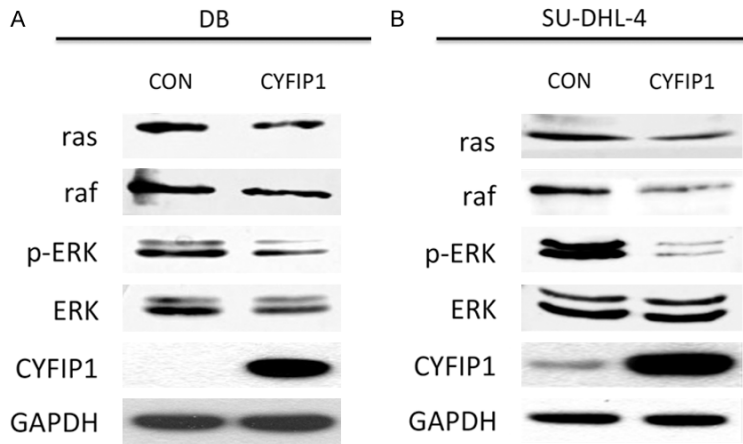


Figure 5. CYFIP1 overexpression suppressed Ras/Raf/ERK signaling. A and B: Ras, Raf, phospho-ERK and total ERK levels were assayed by western blotting in both DB/CYFIP1 and SU-DHL-4/CYFIP1 cells as compared to the control cells.

logarithmic growth phase. Results showed that the expression of Ras, Raf, and phospho-ERK was down-regulated in both DB/CYFIP1 and SU-DHL-4/CYFIP1 cells as compared with their controls (**Figure 5**). Our results demonstrated that Ras/Raf/ERK signaling was suppressed by CYFIP1 overexpression in DLBCL cells.

Discussion

Our study verified a significant reduction of CYFIP1 in human DLBCL both at mRNA and protein levels. In our study, we tested 48 DLBCL tumor samples in which 85.4% (41/48) of the tumor tissues failed to express CYFIP1. The positive CYFIP1 staining in adjacent normal tissues was mostly found in the cytoplasm of vascular endothelial cells and normal lymphocytes. Silva et al. found that the loss of CYFIP1 expression was frequently seen in common human epithelial tumors including colon, lung and breast cancer. In their study, it was reported that up to 59% of colon, 63% of lung and 75% of breast metastases showed negative CYFIP1 expression using immunohistochemistry [7]. Another study indicated that 59.3% of acute lymphoblastic leukemia failed to express CYFIP1 [8]. Impaired CYFIP1 expression was also verified in two human DLBCL cell-lines (DB and SU-DHL-4) compared with normal human peripheral blood lymphocytes. Both mRNA and protein levels of CYFIP1 were significantly lower in DLBCL cell-lines than in normal human lymphocytes. Our observation suggested CYFIP1 in tumors, which was concordant with previous reports [7].

Of the 48 patients studied, we did not find any significant associations between CYFIP1 expression and select clinic-pathological features, which included gender, age, histological type and expression of Bcl-2, Bcl-6, and mum-1. Ki-67 is a nuclear antigen that is expressed in proliferating cells [15]. Expression of Ki-67 is also considered as a useful prognostic factor in various malignancies, including non-Hodgkin's lymphoma [16, 17]. Of seven patients that positively expressed CYFIP1, six of them did not express Ki-67. However, there was no significant correlation between the

expression of CYFIP1 and Ki-67. This might be due to limited sample numbers in our study.

To further explore the function of CYFIP1 in DLBCL, we chose DB and SU-DHL-4 cells with low levels of CYFIP1. We generated and confirmed stable cell-lines with targeted overexpression of CYFIP1 using lentiviral transduction. CYFIP1 expression abundance was significantly increased at both mRNA and protein levels. Thus, CYFIP1 overexpression was specific and efficient, and these stable cell-lines could be used for subsequent studies.

Previous studies found that knock-down of CYFIP1 generated abnormal structures, however, no significant difference was discovered in the levels of proliferation (Ki67) or apoptosis (activated caspase-3) [7]. Furthermore, the influence of CYFIP1 on tumor cells has not been previously reported. In our study, we found that artificial overexpression of CYFIP1 significantly inhibited the growth of DB and SU-DHL-4 cells at 24, 48 and 72 h as compared with the relative control groups. A clear decrease in PCNA levels of two stable cell-lines as compared to the control groups confirmed the inhibitory effect of CYFIP1. Defective apoptosis represents a major causative factor in tumor development and progression [18].

In the present study, induction of apoptosis in stable CYFIP1 over-expressing cells was observed concomitantly with inhibition of cell proliferation. We showed that overexpression of CYFIP1 promoted apoptosis in DLBCL cells,

CYFIP1 is a putative tumor suppressor

which was verified by caspase-3 activation, a signaling executioner in the apoptotic suicide program [19]. In summary, overexpression of CYFIP1 may diminish DLBCL development by inhibiting cell growth and promoting apoptosis. To further investigate the mechanism by which CYFIP1 regulates cell growth, we performed fluorescence-activated cell sorting (FACS) to analyze cell cycle progression, and found that CYFIP1 had little influence on cell cycle. We thus concluded that CYFIP1 inhibited cell growth without inhibiting cell cycle progression.

It is known that phosphorylated ERK (pERK) is a key downstream component of the Ras/Raf/MEK/ERK signaling pathway. After phosphorylation, pERK translocates to the nucleus, where it modulates gene expression and regulates various transcription factors [20]. The Ras/Raf/MEK/ERK pathway plays a pivotal role in cell survival during various stages of cancer [21]. Previous studies proposed that CYFIP1 knock-down cooperates with activated Ras to promote tumor progression [7]. Our results showed that overexpression of CYFIP1 *in vitro* notably decreased Ras, Raf and the phosphorylation of ERK. This observation indicated that the anti-proliferative effect of CYFIP1 was, to a large extent, Ras/Raf/ERK dependent. Oguro-Ando et al. verified that in some forms of Autism Spectrum Disorder (ASD), CYFIP1 overexpression implicates modulation by m-TOR signaling [22]. Our study also found that CYFIP1 overexpression suppressed activation of m-TOR signaling in DB cells; however this observation was not confirmed in SU-DHL-4 cells (data not shown).

Consistent with these observations, we found that the overexpression of CYFIP1 significantly inhibited tumor proliferation and promoted apoptosis in DLBCL cells. Current data supports the tumor suppressive role of CYFIP1 in DLBCL development. Additional studies are required to explore the deeper regulatory mechanisms of CYFIP1 in the progression of DLBCL.

Conclusion

Our findings demonstrate that CYFIP1 expression is decreased in DLBCL. *In vitro*, CYFIP1 overexpression inhibits cell growth and induces apoptosis. To some extent, our observations support the notion that CYFIP1 is a tumor sup-

pressor and it might play an important role in the occurrence and development of DLBCL. It is thus possible that CYFIP1 might serve as a diagnostic marker and potential biomarker in DLBCL.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (No. 81670181), the Youth Fund of Natural Science Foundation (No. 81500118).

Disclosure of conflict of interest

None.

Address correspondence to: Xiaoyan Ke and Lin Fu, Department of Hematology and Lymphoma Research Center, Peking University Third Hospital, Beijing 100191, China. Tel: +86-18810533703; Fax: +86-21-57643271; E-mail: xiaoyank@yahoo.com (XYK); fulin022@126.com (LF)

References

- [1] A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. The Non-Hodgkin's lymphoma classification project. *Blood* 1997; 89: 3909-3918.
- [2] Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, Boldrick JC, Sabet H, Tran T, Yu X, Powell JI, Yang L, Marti GE, Moore T, Hudson J Jr, Lu L, Lewis DB, Tibshirani R, Sherlock G, Chan WC, Greiner TC, Weisenburger DD, Armitage JO, Warnke R, Levy R, Wilson W, Grever MR, Byrd JC, Botstein D, Brown PO and Staudt LM. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 2000; 403: 503-511.
- [3] Sehn LH, Donaldson J, Chhanabhai M, Fitzgerald C, Gill K, Klasa R, MacPherson N, O'Reilly S, Spinelli JJ, Sutherland J, Wilson KS, Gascoyne RD and Connors JM. Introduction of combined CHOP plus rituximab therapy dramatically improved outcome of diffuse large B-cell lymphoma in British Columbia. *J Clin Oncol* 2005; 23: 5027-5033.
- [4] Westin JR and Fayad LE. Beyond R-CHOP and the IPI in large-cell lymphoma: molecular markers as an opportunity for stratification. *Curr Hematol Malig Rep* 2009; 4: 218-224.
- [5] Van Den Neste E, Schmitz N, Mounier N, Gill D, Linch D, Trneny M, Milpied N, Radford J, Ketterer N, Shpilberg O, Duhrsen U, Ma D, Briere J, Thieblemont C, Salles G, Moskowitz CH, Glass B and Gisselbrecht C. Outcome of patients with relapsed diffuse large B-cell lym-

CYFIP1 is a putative tumor suppressor

- phoma who fail second-line salvage regimens in the International CORAL study. *Bone Marrow Transplant* 2016; 51: 51-57.
- [6] Schenck A, Bardoni B, Moro A, Bagni C and Mandel JL. A highly conserved protein family interacting with the fragile X mental retardation protein (FMRP) and displaying selective interactions with FMRP-related proteins FXR1P and FXR2P. *Proc Natl Acad Sci U S A* 2001; 98: 8844-8849.
- [7] Silva JM, Ezhkova E, Silva J, Heart S, Castillo M, Campos Y, Castro V, Bonilla F, Cordon-Cardo C, Muthuswamy SK, Powers S, Fuchs E and Hannon GJ. Cyfip1 is a putative invasion suppressor in epithelial cancers. *Cell* 2009; 137: 1047-1061.
- [8] Chen X, Qin L, Li P and Mo W. Cyfip1 is down-regulated in acute lymphoblastic leukemia and may be a potential biomarker in acute lymphoblastic leukemia. *Tumour Biol* 2016; 37: 9285-9288.
- [9] Swerdlow S, Campo E and Harris NL. WHO classification of tumours of haematopoietic and lymphoid tissues. France: IARC Press; 2008. pp. 2008.
- [10] Hans CP, Weisenburger DD, Greiner TC, Gascoyne RD, Delabie J, Ott G, Muller-Hermelink HK, Campo E, Braziel RM, Jaffe ES, Pan Z, Farinha P, Smith LM, Falini B, Banham AH, Rosenwald A, Staudt LM, Connors JM, Armitage JO and Chan WC. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. *Blood* 2004; 103: 275-282.
- [11] Ke X, Zhao L, Gao Z, Wang J, Dong F, Zhou X, Shi L, Huang X, Jing H, Wang J, Zhao W and Yang XF. Higher rates of t(11;18) in Chinese patients with transformed type of MALT lymphoma suggest novel pathways for progression of the disease. *Leuk Lymphoma* 2007; 48: 2157-2166.
- [12] Dong G, Chanudet E, Zeng N, Appert A, Chen YW, Au WY, Hamoudi RA, Watkins AJ, Ye H, Liu H, Gao Z, Chuang SS, Srivastava G and Du MQ. A20, ABIN-1/2, and CARD11 mutations and their prognostic value in gastrointestinal diffuse large B-cell lymphoma. *Clin Cancer Res* 2011; 17: 1440-1451.
- [13] Chen X, Li P, Yang Z and Mo WN. Expression of fragile histidine triad (FHIT) and WW-domain oxidoreductase gene (WWOX) in nasopharyngeal carcinoma. *Asian Pac J Cancer Prev* 2013; 14: 165-171.
- [14] Jerkeman M, Anderson H, Dictor M, Kvaloy S, Akerman M, Cavallin-Stahl E; Nordic Lymphoma Group study. Assessment of biological prognostic factors provides clinically relevant information in patients with diffuse large B-cell lymphoma—a nordic lymphoma group study. *Ann Hematol* 2004; 83: 414-419.
- [15] Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U and Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* 1984; 133: 1710-1715.
- [16] Szczuraszek K, Mazur G, Jelen M, Dziegiel P, Surowiak P and Zabel M. Prognostic significance of Ki-67 antigen expression in non-Hodgkin's lymphomas. *Anticancer Res* 2008; 28: 1113-1118.
- [17] Dziegiel P, Salwa-Zurawska W, Zurawski J, Wojnar A and Zabel M. Prognostic significance of augmented metallothionein (MT) expression correlated with Ki-67 antigen expression in selected soft tissue sarcomas. *Histol Histopathol* 2005; 20: 83-9.
- [18] Russo A, Terrasi M, Agnese V, Santini D and Bazan V. Apoptosis: a relevant tool for anticancer therapy. *Ann Oncol* 2006; 17 Suppl 7: vii115-123.
- [19] Ashkenazi A. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat Rev Cancer* 2002; 2: 420-430.
- [20] Stirewalt DL, Kopecky KJ, Meshinchi S, Appelbaum FR, Slovak ML, Willman CL and Radich JP. FLT3, RAS, and TP53 mutations in elderly patients with acute myeloid leukemia. *Blood* 2001; 97: 3589-3595.
- [21] Asati V, Mahapatra DK and Bharti SK. PI3K/Akt/mTOR and Ras/Raf/MEK/ERK signaling pathways inhibitors as anticancer agents: Structural and pharmacological perspectives. *Eur J Med Chem* 2016; 109: 314-341.
- [22] Oguro-Ando A, Rosensweig C, Herman E, Nishimura Y, Werling D, Bill BR, Berg JM, Gao F, Coppola G, Abrahams BS and Geschwind DH. Increased CYFIP1 dosage alters cellular and dendritic morphology and dysregulates mTOR. *Mol Psychiatry* 2015; 20: 1069-1078.