Inhibition of JAK2/STAT3 Signaling Pathway Suppresses Proliferation of Burkitt’s Lymphoma Raji Cells via Cell Cycle Progression, Apoptosis, and Oxidative Stress by Modulating HSP70

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Background: The aim of this study was to investigate the effect of the JAK2/STAT3 pathway on the proliferation, cell cycle distribution, apoptosis, and oxidative stress of Raji cells via regulating HSP70 expression.

Material/Methods: Raji cells were divided into Blank, HSP70 siRNA, NC siRNA, AG490 (a JAK2/STAT3 signaling pathway inhibitor), and HSP70 siRNA + rh JAK2 (recombinant human JAK2) groups. HSP70 expression was detected by quantitative real-time reverse transcription-PCR (qRT-PCR); the expression levels of HSP70 and JAK2/STAT3 pathway-related proteins were evaluated by Western blotting; cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays; cell cycle distribution was observed by flow cytometry; cell apoptosis was tested by Annexin V-FITC/PI and Hoechst 33342/PI staining; reactive oxygen species (ROS) production was measured by dichloro-dihydro-fluorescein diacetate (DCFH-DA) assays; and MDA content and SOD and GSH-Px activities were determined using detection kits.

Results: AG490 obviously down-regulated HSP70 expression, inhibited proliferation, induced cell cycle arrest at the G0/G1 phase, and promoted apoptosis in Raji cells; these effects were similar to the effects of HSP70 siRNA. Furthermore, ROS production and MDA content were increased in Raji cells treated with HSP70 siRNA or AG490, while SOD and GSH-Px activities were reduced. Raji cells in the HSP70 siRNA + rh JAK2 group did not significantly differ from those in the Blank group in regards to proliferation, cell cycle, apoptosis, and oxidative stress.

Conclusions: Blocking the JAK2/STAT3 signaling pathway may inhibit proliferation, induce cell cycle arrest, and promote oxidative stress and apoptosis in Raji cells via the down-regulation of HSP70.

MeSH Keywords: Burkitt Lymphoma • HSP70 Heat-Shock Proteins • Janus Kinase 2 • STAT3 Transcription Factor

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Background

Burkitt’s lymphoma (BL), a highly invasive B-cell non-Hodgkin’s Lymphoma (NHL), was initially found in children in central Africa by the British surgeon Denis Parsons Burkitt in 1956 [1,2]. As recorded, children are more susceptible to BL, while adults are more susceptible to large-cell lymphomas, and low-grade B-cell lymphomas predominate [3]. Although there are many prevalent medicines, such as etoposide, prednisone, vincristine, cyclophosphamide, doxorubicin and rituximab [4], that are of great potency in suppressing BL, their unavoidable toxicity prevents their application to the elderly and endemic BL patients in developing countries; moreover, the 5-year event-free survival rate of BL is over 90% in developed countries, while the survival rate is much lower in low-income countries (with a one-year survival rate of only 50%) [5,6]. Therefore, it is urgent to conduct in-depth studies to find more effective therapeutic methods.

Heat shock proteins (HSPs), a conservative group of proteins widely distributed in prokaryotic and eukaryotic cells, are also known as stress proteins (SPs) because they are associated with many other forms of stress [7,8]. According to their molecular weights, HSPs can be classified into 6 families, including HSP100, HSP90, HSP70, HSP60, small molecule HSP (22–23 kDa) and ubiquitin (7–8 kDa) [9]. Among them, HSP70, which functions as a molecular chaperone, is one of the most abundant and best-characterized HSPs [10]. Under adverse environmental conditions, the stress-induced members of the Hsp70 family are reported to be involved in the re-folding and degradation of misfolded proteins [11]. Recently, accumulating evidence has revealed that HSP70 is abnormally over-expressed in many cancers, such as bladder cancer [12], colon cancer [13], and breast cancer [14], to compensate for the basal stress due to nutrient shortage, growth dysregulation and unfolded protein accumulation [15]. A study by Fang X and his team also reported that the blockade of HSP70 may be beneficial to BL patients under chemotherapy treatment [5]. These above findings indicate that HSP70 may play an important role in the pathogenesis and development of tumors, including BL, and it is expected to become an effective target for tumor diagnosis and treatment. In recent years, it has been recognized that HSPs are molecular targets of the JAK/STAT signaling pathway [16]. A previous study reported that the over-expression of HSP70 was closely associated with apoptotic resistance (AR) in pancreatic cancer, which is involved in abnormal JAK2/STAT3 pathway signaling [17]. Moreover, inhibition of the JAK2/STAT3 pathway could reduce the expression of HSP70 and then induce apoptosis and autophagy in primary effusion lymphoma (PEL) cells [18]. To the best of our knowledge, the JAK/STAT pathway is abnormally expressed in many lymphomas, which can lead to the survival/proliferation of cells and tumor immune evasion [19]. However, further investigation is needed to clarify whether the JAK2/STAT3 signaling pathway affects BL cell behavior by regulating HSP70 expression.

In this study, Raji cells were transfected with HSP70 siRNA or treated with AG490 (a JAK2/STAT3 signaling pathway inhibitor)/recombinant human JAK2 (rh JAK2) to explore the effects of the JAK2/STAT3 pathway on the proliferation, cell cycle distribution, apoptosis and oxidative stress of Raji cells via regulating HSP70 expression.

Material and Methods

Cell culture

Raji cells were cultured for serial passaging in RPMI 1640 medium containing N-2-hydroxyethylpiperazine-N’-2-ethane sulfonic acid (HEPES) (25 mmol/L), 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 pg/mL), and L-glutamine (100 mmol/L). In addition, cells were cultured in a humidified incubator with 5% CO₂ and 95% air.

Cell grouping and transfection

Raji cells were divided into 5 groups: Blank group (cells without any treatment), HSP70 siRNA group (cells transfected with HSP70 siRNA), NC siRNA group (cells transfected with negative control siRNA), AG490 group (cells treated with 5μM AG490, the JAK2/STAT3 signaling pathway inhibitor), and HSP70 siRNA + rh JAK2 group (cells treated with HSP70 siRNA and 10 μg/ml recombinant human JAK2). AG490 was purchased from Abmole Bioscience (M1646, Shanghai, China), recombinant human JAK2 was purchased from Thermo Fisher Scientific (PV4393, Rockford, USA), and HSP70 siRNA and NC siRNA were purchased from Santa Cruz Biotechnology (CA, USA). Raji cells collected at the logarithmic growth phase were digested with 0.25% trypsin, prepared into single-cell suspensions, and added to 6-well plates at 2×10⁵ cells/well. When cell confluence reached approximately 90%, cell transfection was performed according to the instructions for the Lipofectamine 2000™ reagent (Invitrogen, San Diego, CA, USA).

Detection of HSP70 mRNA expression by qRT-PCR

Total RNA was extracted from cells using TRIzol reagent (TaKaRa, Shiga, Japan), and the purity, concentration and integrity of extracted RNA were determined using a UV spectrophotometer. The extracted RNA samples were cryopreserved at −80°C for subsequent analysis. Based on the gene sequences published in the GenBank database, the primers were designed using the software Primer3.0 and were then synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Additionally,
reverse-transcription PCR was carried out in accordance with the experimental steps of the reaction kit (Takara, Japan). GAPDH was used as the internal reference, and the relative expression levels of target genes were calculated using the 2−ΔΔCt method. Independent experiments were repeated in triple duplicates.

**Western blotting**

Total protein was analyzed for the protein concentration using a bicinchoninic acid (BCA) kit. The protein samples were added to loading buffer, boiled for 5 min, and loaded onto gels at 60 μg/well. Next, the proteins were isolated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene fluoride (PVDF) membranes and blocked with 5% BSA at room temperature for 1 h. Next, the PVDF membranes were incubated overnight at 4°C with the following primary antibodies: anti-phospho JAK2 (ab32101, 1/5000), anti-JAK2 (ab108596, 1/5000), anti-phospho STAT3 (ab76315, 1/2000), anti-STAT3 (ab68153, 1/2000), and anti-Hsp70 (ab79852, 1/25000); all antibodies were purchased from Abcam (Chicago, IL, USA). The next day, the membranes were washed with TBS plus 0.05% (vol/vol) Tween 20 (TBST) 3 times/5 min, followed by the addition of the corresponding secondary antibody for a 1-h incubation. Later, the membranes were washed again with TBST 3 times/5 min before the chemiluminescence (CL) reaction. -actin was used as the loading control; a Bio-Rad Gel Doc EZ Imager (Bio-Rad, California, USA) was used for development, and Image J was used for the analysis of the gray value of the target bands. Independent experiments were repeated in triple duplicates.

**Detection of cell proliferation by MTT assay**

Raji cells collected at the logarithmic growth phase were made into single-cell suspensions, added to 96-well plates (100 μl/well), and incubated in a 37°C, 5%CO2 incubator for 12 h, 24 h, 36 h, 48 h, and 72 h. Next, 20 μl of MTT solution (5 mg/mL) was added to each well for a 4-h incubation. A microplate reader (Thermo Fisher, Waltham, MA) was utilized to detect the absorbance value (OD) of each well at a wavelength of 570 nm. The experiment was repeated 3 times to obtain the mean OD value.

**Detection of the cell cycle by flow cytometry**

Cells in each group were fixed in iced anhydrous ethanol overnight at 4°C, washed with PBS buffer, and centrifuged at 2000rpm. After removing the supernatant, 500 μl of 1×FACS buffer (containing PBS, 0.1% bovine serum albumin (BSA), and 0.01%NaN3) and 2.5 ml of RNase A (10 mg/ml) were added and thoroughly mixed, followed by incubation for 15 min at room temperature. Next, 25 μl of 1mg/ml propidium iodide (PI) was added, followed by incubation at room temperature for 15 min, avoiding exposure to light. The cell cycle was observed using a FACSCalibur™ Flow Cytometer (Becton Dickinson, Bedford, Mass). The experiments were repeated 3 times.

**Detection of the cell apoptosis rate by Annexin V-FITC/PI staining**

Cells were digested in 0.25% trypsin at 4°C and were centrifuged at 12000rpm for 5 min. After removing the supernatant, the cells were suspended in PBS buffer, and 300 μl of Annexin V-FITC and propidium iodide (PI) were added for 30 min at 4°C, avoiding exposure to light. After incubation in an ice bath, the cells were analyzed for apoptosis using a flow cytometer (BD Pharmingen, San Diego, CA, USA). The lower right quadrant represents early apoptotic cells; the upper right quadrant represents late apoptotic cells; the left lower quadrant represents viable cells; and the upper left quadrant represents necrotic cells. The apoptosis rate=the early apoptosis rate+the late apoptosis rate. The experiments were repeated 3 times.

**Detection of cell apoptosis by Hoechst 33342/PI staining**

Cells were washed twice with PBS buffer, fixed in PBS supplemented with 1% (wt/vol) paraformaldehyde (Fisher Scientific, Pittsburgh, PA), rinsed with tap water, and stained for 30 min with Hoechst 33342/PI (Sigma, St. Louis, MO). Next, the morphologic changes of the nuclei were observed under a fluorescence microscope with a 320-nm to 350-nm filter. The experiment was repeated at least 3 times independently.

**Detection of intracellular reactive oxygen species (ROS) by DCFH-DA assay**

Cells were collected at the logarithmic growth phase, made into a cell suspension of 1×105 cells/ml, added to 6-well plates at 2 ml/well, and incubated for 24 h in an incubator (37°C, 5%CO2). Next, the cells were grouped and administered treatments as described above. After incubation, the upper solution was removed, the plate was washed once with PBS buffer, and serum-free medium containing 10 μmol/L DCFH-DA was added at 200 μl/well to submerge the cells at the bottom. Next, the cells were again incubated for 30 min in a 37°C, 5% CO2 incubator; the plate was washed again with PBS buffer, and the cells were digested with trypsin. Subsequently, the cells were collected, centrifuged for 5 min at 1200 r/min, and washed twice with PBS buffer. Finally, 300 μl of PBS cell suspension was used for detection in a flow cytometer (excitation wavelength, 488 nm; emission wavelength, 525 nm). The experiment was repeated 3 times.
Determination of the intracellular oxidative stress indexes SOD, MDA and GSH-Px

After the density of the cell suspension was adjusted to $1 \times 10^4$ cells/ml, the cell suspension was added to 6-well plates at 2 ml/well, incubated for 24 h in an incubator (37°C, 5%CO$_2$), and randomly divided into groups for subsequent experiments. At the same time, cells were induced for injury and treated with the corresponding drugs as described above. After incubation, the upper solution was carefully removed, the plate was washed twice with PBS buffer, and 500 µl of PBS buffer was added to each well. Next, a liquid transfer gun was used for repeated pipetting and to transfer cells to the EP tube. Next, cells were broken in an ultrasonic processor and centrifuged for 10 min at 10,000rpm, followed by the determination of the total protein concentration, intracellular MDA content, and SOD and GSH-PX activities of the supernatant. The procedures for determination were based on the instructions of the MDA, SOD and GSH-PX kits (Nanjing Jiancheng Bioengineering Institute).

**Statistical analysis**

All statistical data were analyzed using SPSS software. Measurement data are presented as the means± standard deviation (±s). The mean values between 2 groups were compared by independent Student’s t-test, and comparisons among multiple groups were conducted by one-way ANOVA followed by least significant difference (LSD) post-hoc test. $P<0.05$ indicated the difference was statistically significant.

![Figure 1.](image1.png)  
Figure 1. Expression of HSP70 as detected by qRT-PCR (A) and Western blotting (B, C) in Raji cells. * $P<0.05$ compared with the Control group and NC siRNA group; # $P<0.05$ compared with the HSP70 siRNA group.

![Figure 2.](image2.png)  
Figure 2. Expression of JAK2/STAT3 signaling pathway-related proteins in Raji cells as determined by Western blotting. * $P<0.05$ compared with the Control group and NC siRNA group; # $P<0.05$ compared with the HSP70 siRNA group; & $P<0.05$ compared with the AG490 group.
Results

Expression of HSP70 and JAK2/STAT3 pathway-related proteins in Raji cells

Compared with the Control group and NC siRNA group, the other groups showed significantly reduced mRNA and protein expression levels of HSP70. Additionally, the mRNA and protein expression levels of HSP70 were lower in the HSP70 siRNA group than in the AG490 group (all P<0.05). Furthermore, the Blank group was not significantly different from the NC siRNA group regarding the HSP70 expression levels (both P>0.05) (Figure 1). As shown by the results of Western blotting (Figure 2), AG490 down-regulated the expression of p-JAK2 and p-STAT3 (all P<0.05), while cells in the HSP70 siRNA + rh JAK2 group had significantly higher levels of p-JAK2 and p-STAT3 expression than those in the other 4 groups (all P<0.05). Additionally, Raji cells in the Control, NC siRNA and HSP70 siRNA groups were not obviously different from each other regarding the p-JAK2 and p-STAT3 expression levels (all P>0.05), and there were no observable differences in the total protein levels of JAK2 and STAT3 among the groups (all P>0.05).

Effects of the JAK2/STAT3 pathway on Raji cell proliferation and cell cycle distribution by regulating HSP70

As displayed by the MTT assay (Figure 3A), both HSP70 siRNA and AG490 could effectively reduce the proliferation of Raji cells (24 h, 36 h, 48 h, and 72 h) (all P<0.05), and no significant difference was found between the Blank group and NC siRNA group regarding Raji cell proliferation at different time points (all P>0.05). In addition, compared with the HSP70 siRNA group and AG490 group, the HSP70 siRNA + rh JAK2 group had evidently improved cell proliferation (24 h, 36 h, 48 h, and 72 h) (all P<0.05). No observable differences were shown in cell proliferation at 12 h among all groups (all P>0.05). Moreover, HSP70 siRNA and AG490 both induced G0/G1 phase cell cycle arrest (all P<0.05). Compared with Raji cells in the HSP70 siRNA group, cells treated with HSP70 siRNA and rh JAK2 had considerable decreases in the G0/G1 ratio of cells (both P<0.05) (Figure 3B, 3C).

Blocking the JAK2/STAT3 pathway can inhibit HSP70 to promote Raji cell apoptosis

According to the results of Annexin V-FITC/PI staining (Figure 4A), the HSP70 siRNA group (55.2±3.1%) and AG490 group (60.8±4.2%) showed a remarkably higher cell apoptosis rate than the Blank group (14.5±1.3%) and NC siRNA group.
(15.9±1.4%) (all \( P < 0.05 \)), while the HSP70 siRNA + rh JAK2 group (16.3±2.1%) was not significantly different compared to the Blank group and NC siRNA group (all \( P > 0.05 \)). Additionally, Hoechst 33342/PI staining was employed for further evaluation of Raji cell apoptosis (Figure 4B) and showed consistent results with Annexin V-FITC/PI double staining.

**Blocking the JAK2/STAT3 pathway can suppress HSP70 to promote oxidative stress in Raji cells**

According to the results of DCFH-DA assays (Figure 5A), the mean fluorescence intensity (MFI) of ROS in Raji cells was remarkably enhanced after transfection with HSP70 siRNA (all \( P < 0.05 \)), a result that was consistent with the effect of the JAK2/STAT3 signaling pathway inhibitor AG490 (\( P > 0.05 \)). Additionally, Raji cells in the HSP70 siRNA + rh JAK2 group showed obvious reduction in the MFI of ROS compared with the AG490 and HSP70 siRNA groups (both \( P < 0.05 \)). Moreover, with cells in the Blank group as the baseline for comparison, cells in the HSP70 siRNA and AG490 groups had dramatically increased MDA content and had significantly lower SOD and GSH-Px activities (all \( P < 0.05 \)), but those in the HSP70 siRNA and AG490 groups showed no observable differences for these indexes (all \( P > 0.05 \)) (Figure 5B–5D).

**Discussion**

RNA interference (RNAi), which is a highly conserved evolutionary process of post transcriptional gene silencing, refers to the breakage of double-stranded RNA molecules (dsRNAs) into small/short interference RNAs (siRNAs) of 21-23 nucleotides and the subsequent specific degradation of corresponding homologous mRNAs [20,21]. Currently, siRNA has been widely used in the study of genomic function and antiviral therapies [22]. Many researchers have successfully applied RNAi technology to the target silencing of HSP70 in various tumor cells, including HepG2 cells [23] and human leukemia and glioblastoma cells [24]. In this study, the effect of HSP70 siRNA was further confirmed by the results of qRT-PCR and Western blotting, namely, the significant reduction in mRNA and protein expression levels.

An important finding of this study was that HSP70 siRNA can significantly inhibit Raji cell proliferation and cause G0/G1 cell cycle arrest. Similarly, the study by Matokanovic M et al. revealed that chitosan-TPP-HSP70 siRNA could silence HSP70, leading to the down-regulation of BCL-6 and c-Myc, and eventually reducing cell viability and enhancing celastrol-induced anti-proliferation in leukemia and glioblastoma cells [24,25]. Additionally, it was reported that \( \beta \)-mangostin can inhibit the proliferation of human promyelocytic leukemia HL60 cells by inducing G0/G1 phase cell cycle arrest and the down-regulation of the anti-apoptotic genes Bcl-2 and HSP70 [26]. To the best of our knowledge, HSPs are highly conserved stress proteins that can make cells more tolerable to dangerous stresses by promoting cell growth and survival, and HSP70 siRNA may affect cancer cells by shortening the S phase of the cell cycle [27]. In recent years, it has been recognized that HSP70 plays a critical role in the apoptosis of lymphocytes.
For example, Kumar S et al. revealed that chelerythrine could induce apoptosis in Dalton’s lymphoma cells by blocking the expression of HSP70 [28]. In this study, we also found that HSP70 siRNA could effectively promote the apoptosis of Raji cells. Consistent with our finding, another study also reported that cell death induced by the HSP70-specific inhibitor (PES) in primary effusion lymphoma (PEL) was indicated by the appearance of Annexin V/PI double-positive cells [15]. The possible mechanism was that HSP70 can interact with many oncoproteins, such as p53, c-Myc, Akt, cyclin D, Cdk4, and ALK, to affect the pathogenesis and progression of lymphoma [29,30].

Currently, accumulating evidence has proven that the JAK2/STAT3 signaling pathway can participate in the occurrence and development of various tumors, including colorectal cancer [31] and prostate cancer [32]. It is worth noting that gene expression profiling can indicate many intracellular signaling cascades, such as the JAK/STAT pathway, and the pathogenesis of particular subtypes of lymphoma [33]. Coincidentally, we found that the JAK2/STAT3 pathway inhibitor AG490 had the same effect as HSP70 siRNA, suggesting that JAK2/STAT3 was also closely involved in BL. Interestingly, a previous study proved that the JAK2/STAT3 signaling pathway can regulate HSP70 expression to affect the development of diseases. Specifically, Granato M

![Image](https://example.com/image.png)

**Figure 5.** Blocking the JAK2/STAT3 pathway can inhibit HSP70 to promote oxidative stress in Raji cells. (A) Mean fluorescence intensity (MIF) of ROS in each group as measured by the DCFH-DA assay; (B, C) MDA content (B), SOD activity (C) and GSH-Px activity (D) of cells in each group; * P<0.05 compared with the Control group and NC siRNA group; # P<0.05 compared with the HSP70 siRNA group; & P<0.05 compared with the AG490 group.
et al. found that blocking JAK2/STAT3 could reduce the expression of HSP70, thereby down-regulating Mcl-1 expression and inducing apoptosis and the autophagy of PEL cells [18]. By treating BL Raji cells with HSP70 siRNA and recombinant Human JAK2, we also found that the proliferation, cell cycle distribution, and apoptosis of Raji cells did not significantly differ from the cells in the Blank group, further suggesting that the JAK2/STAT3 pathway may affect the pathogenesis and progression of BL by modulating HSP70 expression.

Moreover, an increasing number of studies have proven that the induction of oxidative stress can promote the apoptosis of lymphoma cell [34,35]. Under normal circumstances, the production and clearance of ROS, a product of normal aerobic metabolism, maintain a dynamic balance, but the damage of the cell antioxidant mechanism can cause the accumulation of ROS and result in oxidative stress-induced cell injury [36]. On the other hand, MDA has been widely accepted as an indicator for lipid peroxidation and oxidative stress because its production is closely related to the process of free radical attack on lipids [37]. Additionally, several enzymes commonly found in cells, including GSH-Px and SOD, may play the role of a detoxifying system to prevent damage induced by ROS [38]. In this study, both HSP70 siRNA and AG490 can significantly improve the MFI of ROS and the content of MDA in Raji cells, as well as reduce the activity of SOD and GSH-Px, suggesting that inhibiting HSP70 or the JAK/STAT3 pathway may increase the oxidative stress and induce the apoptosis of Raji cells. In fact, it has been reported that transfection with siRNA targeting HSP70-encoding genes can facilitate the apoptosis of head and neck cancer cells under conditions of oxidative stress [39]. Moreover, Madamanchi NR et al. found a time-dependent increase in HSP70 protein expression in H$_2$O$_2$-induced vascular smooth muscle cells, and this result was reversed after pre-treatment with AG-490, suggesting that HSP70 expression can be regulated via the activation of the JAK2/STAT3 signaling pathway [40]. All the afore-mentioned findings added weight to the hypothesis that blocking the JAK2/STAT3 signaling pathway may promote the apoptosis of BL cells by inhibiting the expression of HSP70.

**Conclusions**

The JAK2/STAT3 signaling pathway inhibitor AG490 can reduce the mRNA and protein expression levels of HSP70 in Raji cells, and blocking the JAK2/STAT3 pathway may suppress the expression of HSP70 and thereby inhibit the proliferation of BL Raji cells, causing G0/G1 phase cell cycle arrest, inducing oxidative stress in cells, and eventually promoting cell apoptosis.

**Conflicts of interest**

None.

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