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The Expression and Significance of *CHFR* in B-cell Lymphoma Cells

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ABSTRACT Objective: To investigate mRNA expression level of *CHFR* gene in lymphoma cells, the effect of *CHFR* gene mathylation and methylation inhibition on Raji cells proliferation and apoptosis. **Methods:** The human Burkitt's Raji lymphoma cells were cultivated *in vitro*. Raji cells were treated in different concentration of methylation inhibitor 5 - Aza-2'-deoxycytidine （5-Aza-dC）, then the expression level change of *CHFR* gene was detected by RT-PCR and methylation of *CHFR* gene was analyzed by MS-PCR. The cells proliferation of Raji cells treated with different concentrations of 5-Aza-dC were analyzed by CCK assay. The apoptosis of Raji cells was analyzed by flow cytometry. **Results:** *CHFR* gene had low-expression in Raji cells. 5-Aza-dC can up-regulate the expression of *CHFR* mRNA by demethylation. The growth of Raji cells was inhibited and the apoptosis was promoted by 5-Aza-dC. **Conclusion:** The expression of *CHFR* can be recovered by the methylation inhibition. *CHFR* gene can inhibit growth in Raji cells, and it may play a negative role during the growth and proliferation.

Key words: CHFR gene; Raji cell; 5-Aza-dC; Methylation; Proliferation; Apoptosis

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Introduction

Lymphoma is one of the most common malignant tumors that originates in the lymphatic system and in recent years, and the incidence of lymphoma has been increasing[1, 2]. The initial treatment of Lymphoma is chemotherapy. However, the toxic and side-effect of chemotherapy and the generation of tumor multidrug resistence have been primary factors of affecting long-term free survival rates. So it is very important to find a more effective treatment for improving the rate of complete remission with disease free survival. Malignant tumor is a pathological process involving multiple genes which are mainly oncogenes,tumors suppressor genes and DNA mismatch repair genens.

CHFR gene which was discovered in the year 2000 by Scolnick and Helazonetis is a tumors suppressor gene and localized to chromosome 12q24.33. CHFR gene encodes a 664 amino acids protein which has forkhead-associated domain (FHA), ring finger domain (RF) and cysteine-rich region that govern transition from prophase to metaphase in the mitotic checkpoint pathway. CHFR prevents errors in chromosome segregation which can lead to neoplasia. CHFR is ubiquitously expressed in normal human tissues, while the loss of CHFR expression has been observed in human tumors, in which it fails to prevent proliferation of abnormal cells from G2 to M phase, then abnormal differentiation an proliferation of cells occurs[3]. Loss of gene expression may occure through several different mechanisms. The DNA promoter methylation seems to be one of the most common epigenetic events [4-6], as it has been shown in the laryngeal squamous cell carcinoma tissue (22%)[7], hepatocellular carcinoma (30%)[8], lung adenocarcinomas (10%)[9], and in some other human neoplasia tissue and cell lines. However, the report of CHFR gene expression and methylation in lymphoma was rarely. Our previous experiments have confirmed that the CHFR gene expression was down-regulation in B-cell Non-Hodgkin's lymphoma (B-NHL), and aberrant methylation of CHFR was a frequent event in B-NHL [10]. This study find CHFR gene can inhibit growth and promoting apoptosis in Raji cells, which may contribute to the occurrence of B-NHL, and it aimed to investigate the relationship between CHFR gene expression and lymphoma, and to find a new idea of molecule marks and therapy target in clinical practice.

1 Materials and methods

1.1 Materials and cells

Raji cell line(the type culture collection of the Chinese acade-

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my of science, Shanghai, China) were cultured in RPMI1640 medium (Solarbio), supplemented with 10% FCS (Si jiqing, Hangzhou). Peripheral blood of Non-cancer patients were obtained Non-cancer volunteers, Isolation Reagent(BioTeke, Beijing), CCK (7sea Pharmatech Co., Ltd), 5-Aza-2'-deoxycytidine (sigma), Rt Kit (TaKaRa Co., Ltd), PBS (Solarbio, Beijing), DNA Isolation Reagent (BioTeke), Annexin V-FITC/PI apoptosis assay kit (Zoman biotechnology), Wizard DNA clean-up system (Promega, Madison, WI, USA).

1.2 RNA extraction and RT–PCR(Reverse–transcriptase PCR)

The primer sequences of CHFR were 5'-3' (forwards) GTGTCGCCCCTCAGCAGTGTG, (reverse) CAGTAGCAGTCAGGACGGGAT (GenBank accession nnumber:NM018223), products size 400bp. AT the same time, the GAPDH (GenBank accession number:J04038) whose primer sequences were 5'-3' (forwards) TGATGACATCAAGAAGGTGGTGAAG, (reverse) TCCTTGGAGGCCATGTGGGCCAT was amplified,products size 240bp. The CHFR gene and GAPDH primers were designed according to the relevant citation [11]. Total RNA We extracted from Raji cells and peripheral blood of Non-cancer patients according to RNApure, respectively. The first-strand cDNA was generated and amplified CHFR gene with primers above according to Rt Kit. PCR amplification consisted of 1 cycle at 94 ℃ for 5 min Initial denaturation, 30 cycles at 94 ℃ for 30 s denaturing, 56 ℃ for 45 s annealing and 72 ℃ for 6min extension. The amplified products were separated on a 2% agarose gel electrophoresis and analysed by an imaging system.

1.3 5–Aza–dC treatment

To detect the restoration of CHFR gene expression, the Raji cells were treated with different concentration 1, 4, 7, 10 μmol/L of 5-Aza-dC. The medium and drug were replaced every 48h. After culturing those Raji cells above for 4 days, RNA was extracted for RT-PCR. The amplified products were separated on a 2% agarose gel electrophoresis and analysed by an imaging system.

1.4 Bisulfite treament and methylation–specific PCR (MSP)

Genomic DNA was isolated from Raji cells and normal human peripheral blood according to DNA Isolation Reagent. Briefly, 3 μg aliquots of Raji cells genomic DNA was dilutedr to 50 microliters with ultrapure water, then which was denatured by 5.5 μL of 3mol/l NaOH for 30min at 42 ℃ and modified by 520 μL of 3.6 mol/l sodium bisulfite and 30 μL of 10mmol/l hydroquinone for 16 h at 50 ℃. DNA was purified by using the Wizard DNA clean-up system, treated again with NaOH and recovered in ethanol and resuspended in 25 μL distilled water. Normal human peripheral blood genomic DNA which has been methylated modification by SssI was used as a positive control for methylated DNA. Distilled water without DNA was used as a negative control for non-methylated DNA. MS-PCR was carried out with the following oligonucleotide primer, which were designed to be specific to either methylated or unmethylated DNA after sodium bisulfite modification as described above.

According to the relevant citation [12], the unmethylated DNA-specific primer were 5'-3'TTTTGTGATTTGTAGGTGAT (forward), 5'-3' (reverse)ACAATTAACTAACTAACAACAACA, products size 155bp, and methylated DNA-specific primer were 5'-3' (forward)TTTCGTGATTCGTAGGCGAC, 5'-3' (reverse)GCGATTAACTAACGACGACG, products size 155bp. MS-PCR amplification consisted of 95 ℃ for 5 min Initial denaturation, 95 ℃ for 30sec, 58 ℃ for 30sec (Methylated DNA), or 53 ℃ for 30sec (unmethylated DNA), 72 ℃ for 45sec, 30 cycles, and 72 for 10 min. The amplified products were separated on a 2% agarose gel.

1.5 CCK assay

1× 10^5 cells were plated into every well of 96-well plates and cultured these cells with 1, 4, 7 and 10 μmol/L of 5-Aza-dC for 24h, respectively, and the control group was treated with isopyknic PBS and the Bank group contained only isopyknic medium with5-Aza-dC, after which we added 10 μL CCK-8 to every well. Then these cells were incubated for 4h at 37 ℃ in 5% CO_2. Optical density was analyzed at 450nm by using microplate Readers. Cells Inhibition rate=1- (Experimental groupOD450-Bank group OD450)/ (control group OD450-Bank groupOD450).

1.6 Flow cytometry assay

Raji cells were plated into 6-well plates and treated with 1, 4, 7 and 10 μmol/L of 5-Aza-dC and the control group was only treated with drug-free medium for 24 h, and then washed with PBS. 1× 10^6 single-cell suspensions were prepared and treated according to Annexunv/FITC Rt, after which they were cultured in the dark at room temperature for 15min. Flow cytometric detected early apoptosis rate by the way of AnnexinV/PI in an hour.

1.7 Statistical Analysis

Data were analyzed by the SPPS17 Software. Correlation of every group was assessed by One way Anov. A value of $P < 0.05$ was considered statistically significant.

2 Results

2.1 CHFR gene expression and re–expression analysis in drug–free Raji cells and 5–Aza–dC treatment group by using RT–PCR

The expression of CHFR gene was detectable in the human normal peripheral blood cells, while in Raji cells, we only can detect the weak expression of CHFR gene (Fig.1). The CHFR gene was recovered to express after the treatment of 5-Aza-dC in a concentration-dependent manner. With the elevation of the drugs concentration, the expression of CHFR gene mRNA was increased (Fig.2).

2.2 Analysis of CHFR gene methylation in Raji cells by MS–PCR

The Raji cells showed partial methylation. After the treatment of 5-aza-CdR, With the elevation of the drugs concentration, the methylation states were decreased and unmethylation states were increased.(Fig.3).

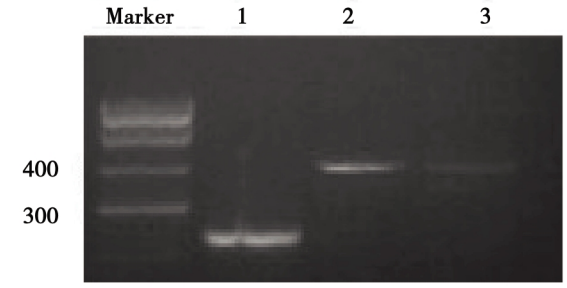

Fig.1 The expression of the CHFR gene in Raji cells and the human normal peripheral blood

1: GAPDH; 2: The human normal peripheral blood; 3: Raji cells

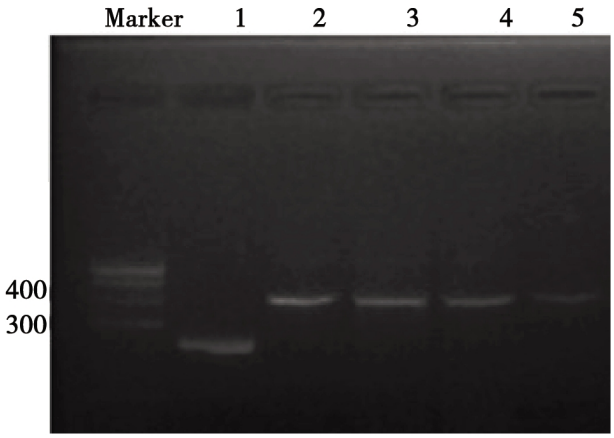

Fig. 2 Effect of 5-Aza-dC on methylation of CHFR gene

1:GADPH; 2: 10 μmol/L 5-Aza-dC; 3: 7 μmol/L 5-Aza-dC; 4: 4 μmol/L 5-Aza-dC; 5: 1 μmol/L 5-Aza-dC

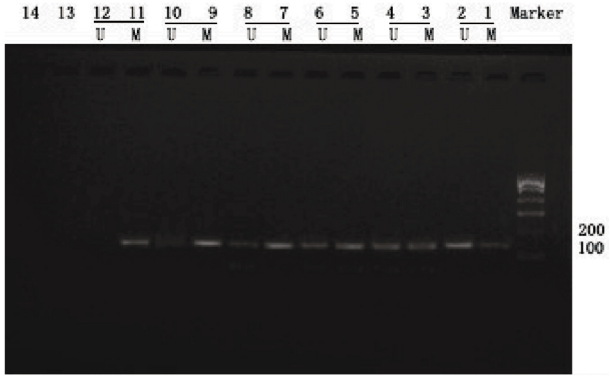

Fig.3 MSP analysis the methylation status of the CHFR gene

13, 14: Bank; 11, 12: positive control; 9, 10: drug-free control; 7, 8: 1 μmol/L 5-Aza-dC; 5, 6: 4 μmol/L 5-Aza-dC; 3, 4: 7 μmol/L 5-Aza-dC; 1, 2: 10 μmol/L 5-Aza-dC.

2.3 Analysis of Raji cells inhibition rate after treatment with 5-Aza-dC by CCK

The inhibition rate of these cells which have been treated with different concentrations of 5-Aza-dC was (2.6 ± 0.40)%, (8.8± 0.96)%, (12.4± 0.61)%, (16.2± 1.1)%. There was statistically significant difference between these groups ($P < 0.05$). With the elevation of the drugs concentration, the inhibition rate increased.

2.4 Analysis of Raji cells apoptosis rate after treatment with 5-Aza-dC by Flow cytometry assay

The early apoptosis rate of Raji cells in the control group was (1.72± 0.1)%. These cells which were treated with 5-Aza-dC were (3.86± 0.35) %, (6.27± 0.4)%, (9.57± 0.33)%, (15.5± 0.46)%. There was statistically significant difference between these groups ($P < 0.05$). With the increased of 5-Aza-dC concentrations, the apoptosis rate of Raji cells increased. (Fig.4).

3 Discussion

CHFR is a recently identified gene with FHA, ring finger and cysteine-rich (CR) domains, which functions as an important checkpoint protein early in G2/M translation, and its activation delays the cell cycle in prophase, thus preventing chromosome condensation in response to mitotic stress [13]. Both the FHA and CR domains are required for its checkpoint function [13]. The RF domain of CHFR gene confers ubiquitin ligase (E3) activity and plays a critical role in the ubiquitination of substrates[3]. Some studies suggested that CHFR regulate the prophase checkpoint and maintain genomic stability to inhibit tumorigenesis by regulating multiple mechanisms, including inhibition of accumulation of cyclin B1 in the nucleus, degradation of PLK1 and Aurora-A, and inhibition of Cdc2 kinase activity to ensure the accuracy of mitotic[3, 14-16].

At present, numerous studies have showed that the loss of CHFR mRNA expression and the promoter aberrant methylation of CHFR gene occurred frequently in many human cancer cells but rarely in the adjacent normal mucosa and tissue, for example oral squamous carcinomas[17], gastric carcinoma[18], hepatocellular carcinoma [8]. These studies showed that the epigenetic inactivation of CHFR plays an important role in human cancer, while inactivation of the gene may be caused by DNA methylation. Methylation modification plays an important role in the gene expression regulation and genetic structure stabilization. The decreased or lost CHFR expression may lead to genomic instability by impairing the spindle assembly checkpoint, which can lead to cells a high mitotic, suggesting that CHFR gene function as a tumor suppressor gene [16,19-21].

Some studies suggested that treatment with DNA methylation inhibitor can restore the activities of the CHFR gene and decrease the growth rate of cancer cells and promote cancer cells apoptosis, which makes it possible to find a useful molecular maker for predicting the sensitivity to particular chemotherapeutic agents [7, 22-24]. Currently, decitabine, a methylation inhibitor, has been shown efficacy in some clinical trials [25-27], such as MDS, Leukemia, Non-Hodgkin's lymphoma.

This study found CHFR gene expression had down-regulation and 5-Aza-dC could restore the activities, inhibit the growth and promote apoptosis in Raji cells. In conclusion, CHFR gene plays a negative role during the growth and proliferation of Raji cells. Methylation inhibitor may be used as a molecular target to cure the lymphoma, and CHFR gene might be a therapeutic target against lymphoma in the future.

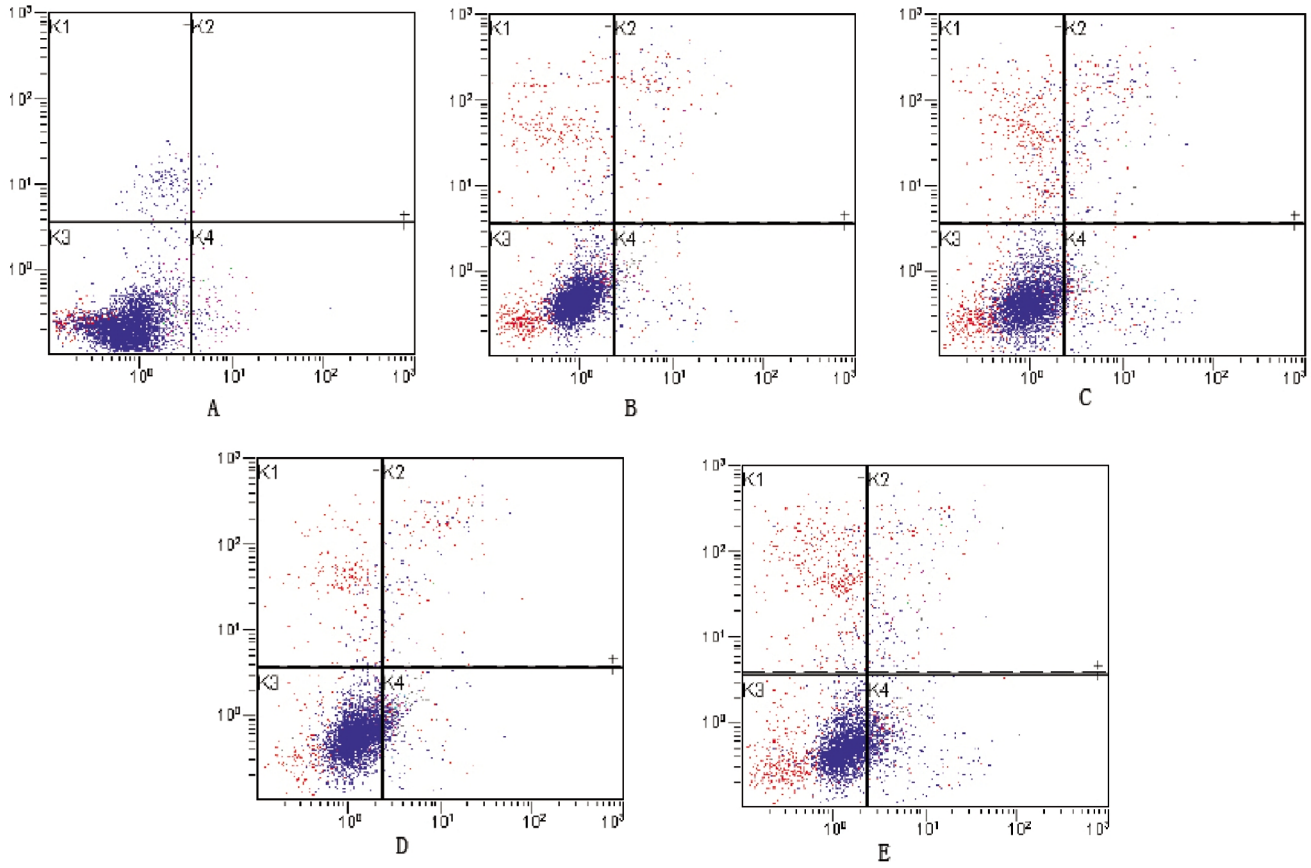

图 4 Analysis of Raji cells apoptosis rate by Flow cytometry

A: drug-free control; B: 1 μmol/L 5-Aza-dC; C: 4 μmol/L 5-Aza-dC; D: 7 μmol/L 5-Aza-dC; E: 10 μmol/L 5-Aza-dC.

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CHFR 基因在 B 细胞淋巴瘤细胞中的表达及意义

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摘要 目的：探讨 *CHFR* 基因在 B 细胞淋巴瘤 Raji 细胞中的表达，以及 *CHFR* 基因甲基化对 Raji 细胞增殖和凋亡中所产生的影响。**方法**：体外培养人 B 细胞淋巴瘤细胞株 Raji 细胞，用不同浓度的去甲基化试剂 5- 氮杂 -2 脱氧胞苷处理 Raji 细胞株，通过 RT-PCR 检测 CHFR 基因表达水平的变化，通过 MS-PCR 检测 *CHFR* 基因甲基化变化，CCK 法及流式细胞术检测 Raji 细胞增殖及凋亡变化。**结果**：*CHFR* 基因在 Raji 细胞中出现弱表达，经去甲基化试剂处理后 *CHFR* 基因表达水平增高，随药物浓度增加 Raji 细胞的抑制率及凋亡率增高。**结论**：5- 氮杂 -2 脱氧胞苷可以恢复 *CHFR* 基因表达水平，抑制 Raji 细胞的增殖，促进凋亡。*CHFR* 基因在细胞增殖起负向调控的作用。

关键词：*CHFR* 基因；Raji 细胞；5- 氮杂 -2 脱氧胞苷；甲基化；增殖；凋亡

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