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Cholesterol synthesis is essential for central nervous system leukemia cell survival

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Abstract

Central nervous system leukemia (CNSL) is a common complication of leukemia, especially for acute lymphoblastic leukemia (ALL), which seriously affects the CNS and prognosis of patients. At present, because the mechanism for CNSL cell survival is not clear, targeting and low-toxic treatment approaches are still lacking. In this study, we found that cholesterol synthases expression is up-regulated in leukemia cells of cerebrospinal fluid (CSF) as compared to bone marrow by using clinical specimens and GEO database. Simvastatin treatment or using delipidated FBS medium to simulate the lipid-deficient microenvironment resulted in leukemia cell survival inhibition. In summary, we demonstrate cholesterol synthesis is essential for CNSL cell survival, targeting cholesterol synthases is a promising approach for CNSL treatment.

Keywords

CNSL, ALL, cholesterol synthesis, cell survival.

1. Introduction

Acute lymphoblastic leukemia (ALL) is a common disease in children with hematological malignancies. A large number of abnormally proliferating leukocytes not only cannot exercise immune function like normal lymphocytes, but also inhibit normal hematopoietic function. In addition, a large number of abnormally proliferating leukocytes will invade various organs through blood circulation[1].The central nervous system (CNS) is one of the most common sites of leukemia cells invasion. Central nervous system leukemia (CNSL) is a clinical symptom caused by extramedullary infiltration of leukemia cells into the arachnoid membrane or its adjacent nervous tissue outside the bone marrow, mainly manifesting as focal or diffuse infiltration of leukemia cells in the meninges and parenchyma, which may be accompanied by hemorrhage, hematoma and epidural mass to form transverse myelitis, and is also one of the main causes of recurrence of acute leukemia, especially ALL. Once it occurs, it will seriously affect the prognosis of patients[2].

As a complication of ALL, CNSL is seriously harmful to the nervous system of the ALL patients, but the mechanism of CNSL occurrence and progression has not been fully elucidated to date. The current methods for treating CNSL, such as intrathecal injection of anti-hematological malignancy drugs, intracranial irradiation and high-dose systemic chemotherapy, although they induce certain therapeutic effects, there are acute or long-term severe neurotoxicities[3]. Most chemotherapeutic drugs do not easily cross the blood brain barrier (BBB) , resulting in no effective drug concentration in the cerebrospinal fluid (CSF), and the invading leukemic cells slowly proliferate, ultimately leading to CNSL[4]. Because there is a lack of understanding of the mechanism of CNSL cells survival, it is difficult for us to propose targeted and low-toxicity treatments.

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This study is dedicated to investigate the role of cholesterol synthases in the survival of CNSL cells from a new perspective of cholesterol synthesis to provide new targets and ideas for CNSL treatment.

2. Materials and methods

2.1. Cells

The clinical specimens used in this study were provided by Nanfang Hospital, Guangzhou, and leukocytes were obtained by lysing red blood cells from BM specimens with red blood cell lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$, 1 mM Na_2EDTA), and white precipitates obtained by CSF specimens were leukemia cells.

The T-ALL cell lines Molt-4 and Jurkat used in this study were cultured in RPMI1640 medium (Gibco™) containing 10% FBS (Ausgene™), 100 U/ml penicillin and 100 mg/ml streptomycin (HyClone™). The humidified cell incubator temperature was maintained at 37°C and the CO_2 concentration was maintained at 5%.

2.2. Quantitative RT-PCR

Cellular total RNA was extracted with TRIzol reagent (Invitgen™) according to the manufacturer's instructions. The gDNA removal and reverse transcription reactions of total RNA sample were performed using Evo M-MLV reverse transcription premixed kit (AgBio®). The RT-PCR reagents system was formulated using SYBR PreMix ex Taq (Takara®), followed by quantitative RT-PCR on a Bio-rad® CFX96 fluorescent quantitative PCR system. Relative quantitative analysis was finally performed using the ΔΔcycle threshold method, and β-Actin was used as an endogenous control to normalize expression levels. Relative gene expression was presented as log ($2^{-\Delta\Delta}$ cycle threshold). The primer sequences are as following (**Table 1**):

Table 1 Primer sequence for quantitative RT-PCR

Gene	Sense	Anti-sense
SREBP2	5'-GGCTCCAACTCTGCAAGTCAAGG-3'	5'-GTCTGCGGAGTGGTGCTGAATG-3'
HMGCS1	5'-GATGTGGGAATTGTTGCCCTT-3'	5'-ATTGTCTCTGTCTCTCCAG-3'
HMGCR	5'-TCTGGCAGTGGGAACTATT-3'	5'-CCTCGTCCTTCGATCCAATTT-3'
NSDHL	5'-GGAGCGAGGCTATACTGTCAATG-3'	5'-TTACACCTTTGAGAGCTGGGTACA-3'
SQLE	5'-GATGATGCAGCTATTTTCGAGGC-3'	5'-CCTGAGCAAGGATATTCACGACA-3'
MVD	5'-TATGCCTGCCTAGCCTACAC-3'	5'-CCCATCTGCCACTCCACAAA-3'
MVK	5'-GTACCTCGTGCTGGAAGAGC-3'	5'-GCTTGAGGAGTGTGATGCCA-3'
SQS	5'-GCAACGCAGTGTGCATATTTT-3'	5'-CGCCAGTCTGGTTGGTAAAGG-3'
β-Actin	5'-GCCGCCAGCTCACCAT-3'	5'-TCGTCGCCCACATAGGAATC-3'

2.3. GEO database analysis

By searching for datasets related to ALL and CNSL in the GEO database (https://www.ncbi.nlm.nih.gov/geo/), the data of GSE60926 and GSE135115 were downloaded and the expression levels of different samples were analyzed. Using Graghpad Prism 8.0 software for plotting and conducting a wilcox test on the expression of cholesterol synthases in different groups of samples, $P<0.05$ indicates significant differences.

2.4. CCK-8 assay

Cell Counting Kit-8 (Dojindo®) was used to detect cell viability. Briefly, the cells to be tested were seeded into 96-well plates at a concentration of 40,000 cells per well at different time points, 10 μL CCK-8 solution was added to each well, incubated at 37 ° C / 5% CO_2 humidified cell incubator for 1- 4 h, and the absorbance of the 96-well plate was measured at 450 nm on a BMG LABTECH® microplate reader. In this experiment, test well As, control well Ac and blank well Ab were generally set up, and the cell proliferation rate = [(As-Ab)/(Ac-Ab)] × 100%.

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2.5. Lipid-deficient microenvironment simulation

The delipidated FBS (Gemini™) was used to replace conventional FBS and to prepare the delipidated culture medium to simulate the lipid-deficient microenvironment of CNSL survival.

2.6. Statistical analysis

The data were processed using Graghpad Prism 8.0 software and presented as mean ± SD from at least three independent experiments, and comparisons between groups were performed by unpaired two-tailed Student's t test (except 2.3 GEO database analysis), which were statistically significant when P<0.05.

3. Results

3.1. Up-regulation of cholesterol synthases expression in CNSL cells was confirmed in clinical specimens

Leukemic cells were obtained by collecting BM and CSF clinical samples from B-ALL and T-ALL patients, and the expression levels of cholesterol synthases SREBP2, HMGCS1, HMGCR, NSDHL, SQLE, MVD, MVK and SQS (Fig. 1A) in these two types of cells were quantitatively determined by RT-PCR, in which HMGCR (3-hydroxy-3-methyl glutaryl coenzyme A reductase) and SQLE (squalene epoxidase) are the first and second rate-limiting enzymes of cholesterol biosynthesis, respectively[5]. The results show that cholesterol synthases expression is significantly up-regulated in CSF-derived leukemia cells compared with BM-derived leukemia cells ($P < 0.001$) (Fig. 1B, C).

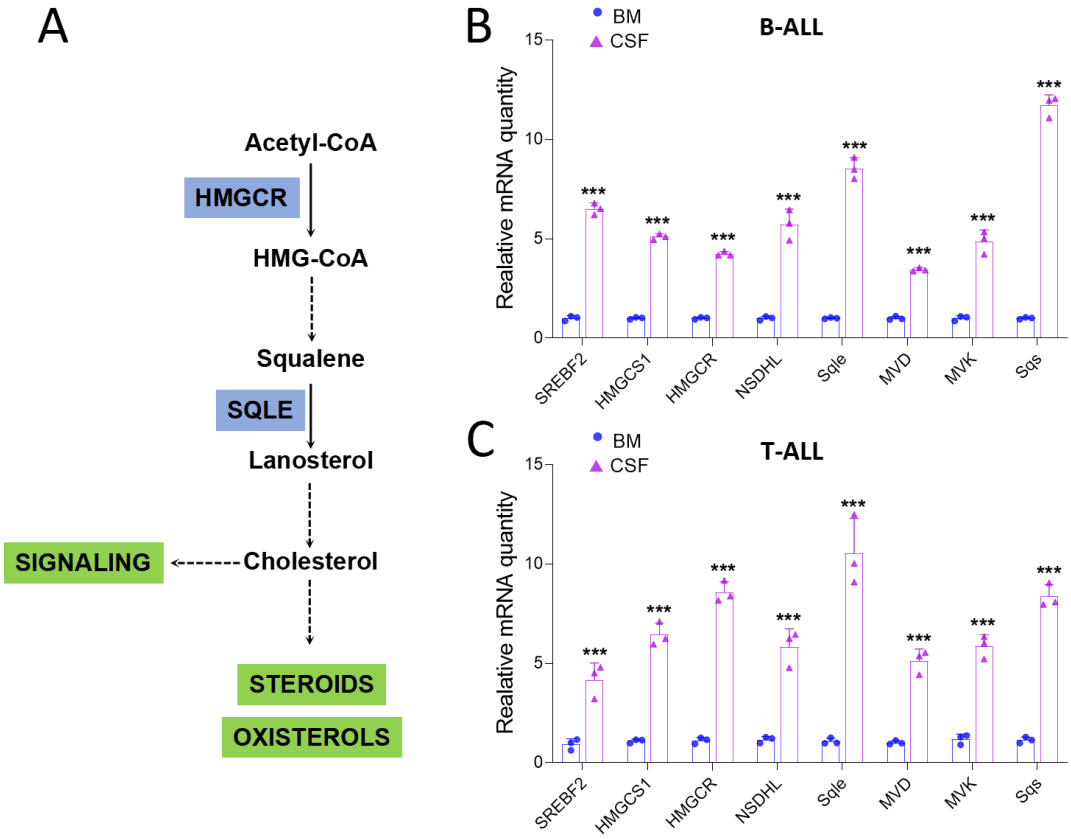

Fig. 1 Expression of cholesterol synthases is significantly up-regulated in CSF-derived leukemic cells. (A) Schematic diagram of cholesterol biosynthesis; (B, C) RT-PCR quantitative detection of cholesterol synthases expression levels in leukemia cells from BM and CSF of B-ALL and T-ALL patients (***P < 0.001)

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3.2. GEO database analysis confirms up-regulation of cholesterol synthases expression in CNSL cells

To further demonstrate the up-regulation of cholesterol synthases in CNSL cells, gene expression data from the RNA Expression Chip Database (NO.GSE60926) were downloaded and analyzed from the GEO database, which contained BM samples from 42 patients with ALL and CSF samples from 8 patients with ALL accompanied by CNSL. It is found that the cholesterol synthases expression of SREBF2, HMGCS, HMGCR and SQS in CNSL cells are significantly higher than that in BM-derived cells (P<0.001) (Fig. 2A,B). Analysis of an additional constructed RNA expression sequencing database of the CNSL mouse model (NO. GSE135115) also shows similar results: cholesterol synthases expression is up-regulated in CNSL cells compared with spleen-derived leukemia cells (Fig. 2C, D). These results confirmed that CNSL cells up-regulate cholesterol synthases expression as compared to bone marrow and spleen.

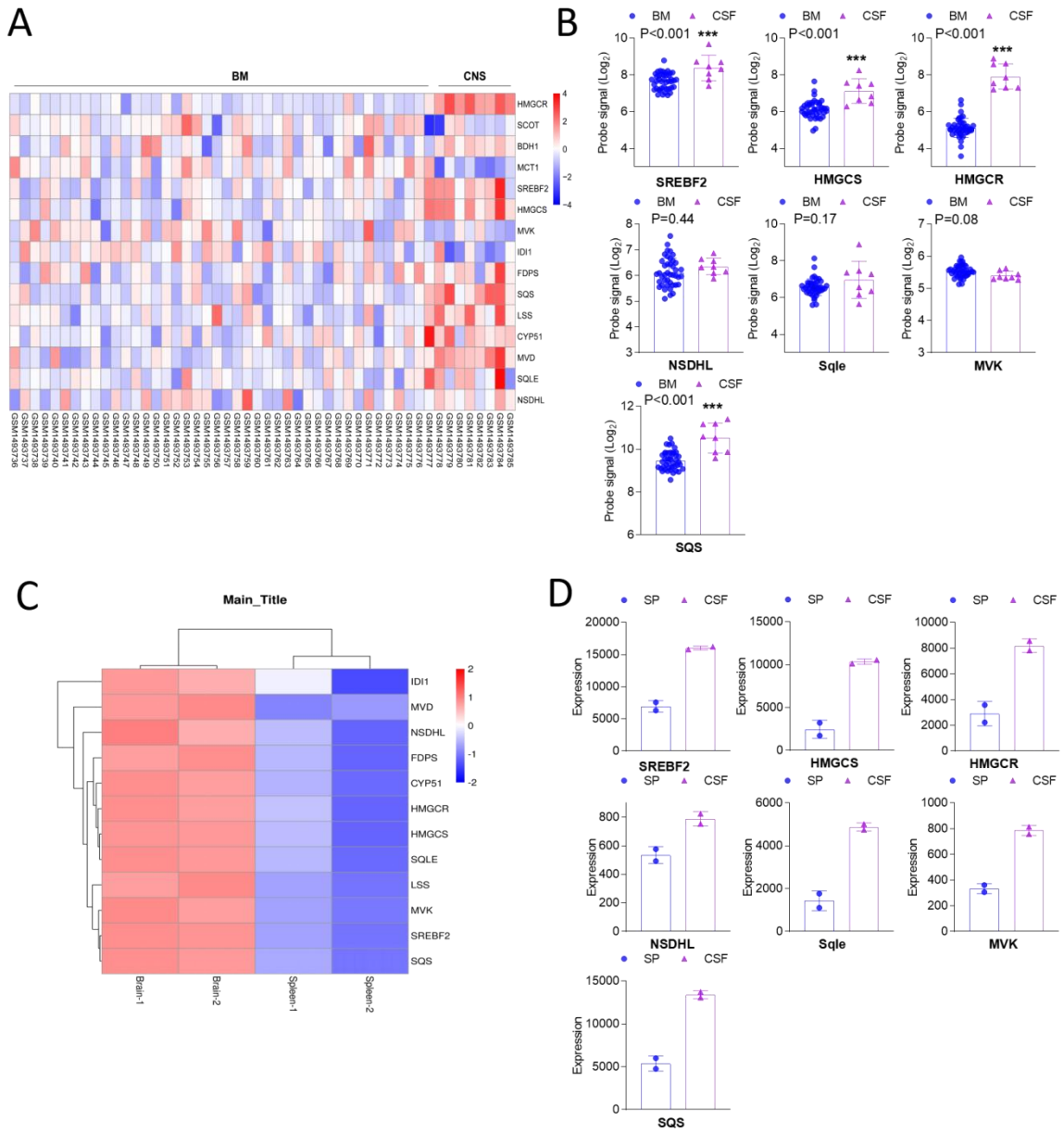

Fig. 2 GEO database analysis of cholesterol synthases expression levels in CNSL cells. (A, B) GSE60926 dataset analysis of relevant gene expression levels in BM samples (A) from ALL patients and CSF (B) samples from ALL patients with CNSL; (C, D) GSE135115 dataset analysis of relevant gene expression levels in spleen-derived and CSF-derived leukemic cells(***P<0.001)

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3.3. Cholesterol is required for survival of T-ALL cells

To determine the role of cholesterol synthesis in maintaining leukemia cells survival, T-ALL cell lines were first treated with the *Simvastatin* to reduce cellular cholesterol[6]. Cell proliferation was detected by CCK-8, and it was found that inhibition of cholesterol synthesis significantly decreased Molt-4 (Fig. 3A) and Jurkat (Fig. 3B) proliferation (P<0.001). Using delipidated FBS medium to simulate the lipid-deficient microenvironment, it is found that delipidated FBS culture inhibited proliferation of T-ALL cells, but additional cholesterol supplementation may rescue it (P<0.001) (Fig. 3C, D). It demonstrates that cholesterol is required for T-ALL cell survival maintain.

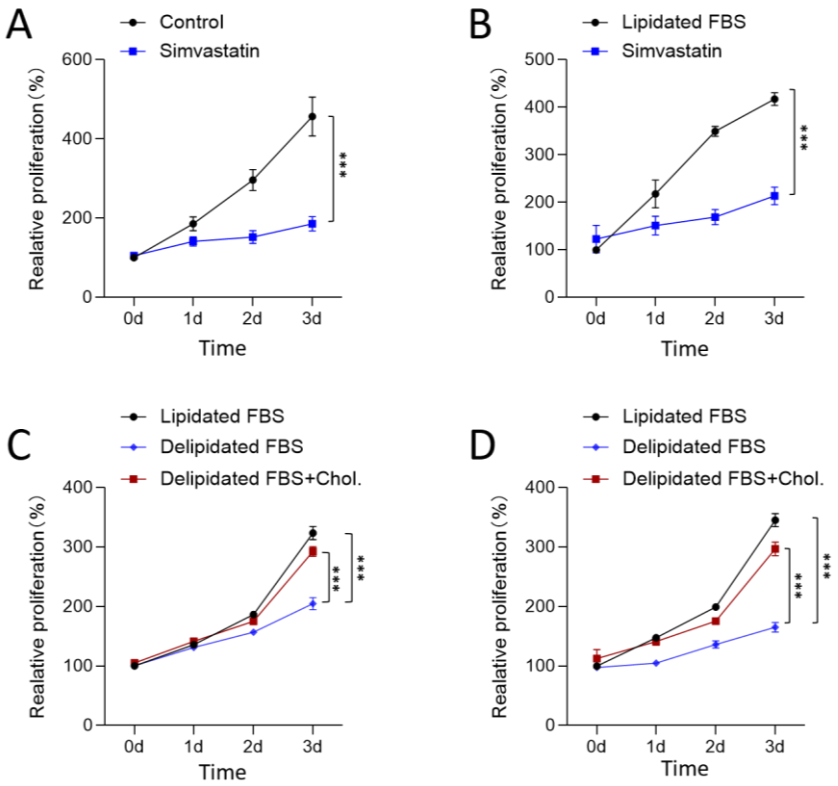

Fig. 3 Cholesterol deficiency Inhibits T-ALL cells proliferation. Cell proliferation after *Simvastatin* treatment of Molt-4 (A) and Jurkat (B) cells; Cell proliferation in the presence of lipid deficiency and additional cholesterol supplementation of Molt-4 (C) and Jurkat (D) cells (***$P < 0.001$)

4. Discussion

In summary, up-regulation of cholesterol synthases expression in CNSL cells is confirmed by gene expression analysis of cells from ALL clinical specimens and GEO database; secondly, cholesterol is confirmed to be required to the survival of T-ALL cells by Simvastatin treatment as well as simulating a lipid-deficient microenvironment, and stimulated by HMGCR, SQLE and other synthases critical for inducing cholesterol synthesis in T-ALL cells, are up-regulated and also cholesterol synthesis is promoted for maintaining the leukemia cell survival.

Uncontrolled proliferation of cancer cells require the accumulation of large amounts of lipids which can be acquired from exogenous sources or synthesized endogenous through the lipogenic pathway to constitute the membranes and organelles. Cholesterol metabolism balance is essential to maintain the vital movement, which plays an important role in cell growth, proliferation, differentiation, survival, apoptosis, inflammation, motility and cell membrane homeostasis[7]. Compared with normal cells, cholesterol synthesis is up-regulated in tumor cells, and cholesterol metabolism is widely involved in malignant proliferation,

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migration, and invasion of tumor cells, and eliminating cholesterol or hindering cholesterol transport can inhibit the growth and invasion of a variety of tumors[8, 9]. Little or no cholesterol is transported from the peripheral circulation into the CNS due to the presence of the blood-brain barrier (BBB). Thus, cholesterol required in the CNS is essentially derived from endogenous synthesis. CSF is a secreted product of the CNS, and in this microenvironment, leukemic cells infiltrate the CSF of leptomeninges and are also trapped in this nutrient-deficient area due to BBB[10], and how CNSL cells maintain survival here deserves our investigation. It has previously been pointed out that CNSL cells undergo aliphatic acid anabolic remodeling to adapt to the nutrient-deficient microenvironment of CSF, up-regulate the expression of key enzymes of aliphatic acid synthesis, and maintain survival by synthesizing aliphatic acid themselves, while inhibitors targeting fatty acid synthesis can effectively alleviate the occurrence of CNSL[11]. Proliferation and survival of leukemic cells requires a large number of nutrients including cholesterol, but so far, the mechanism of how leukemic cells maintain survival in this nutrient-deficient microenvironment of CSF after transferring from nutrient-rich peripheral tissues such as spleen and bone marrow (BM) to the CNS has not been reported. We speculate that when leukemia cells invade the CNS, they will up-regulate their key synthases expression for cholesterol de novo synthesis, and drive cholesterol anabolism to adapt to the nutrient-deficient microenvironment of CSF, thus maintaining long-term survival themselves. Cholesterol synthases such as HMGCR and SQLE are considered to be potential targets for the clinical treatment of CNSL.

Acknowledgements

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