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Downregulation of GLUT3 Promotes Apoptosis and Chemosensitivity of Acute Myeloid Leukemia Cells via EGFR Signaling

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Abstract

Background: Glucose transporter 3 (GLUT3) plays an important role in tumor progression and drug resistance in numerous malignancies, including acute myeloid leukemia (AML). However, the effect of GLUT3 silencing on treatment of AML remains poorly understood. The purpose of this study was to investigate role of GLUT3 in proliferation and chemosensitivity of AML and its underlying mechanisms.

Methods: The siRNA transfection was conducted using Lipofectamine™ 2000. Quantitative real-time RT-PCR (qRT-PCR) and Western blot analyses were employed to measure the expression levels of mRNA and protein for GLUT3, respectively. The cytotoxic effects of siRNA and vincristine were determined using the MTT assay. Flow cytometry was performed to analyze apoptosis.

Results: GLUT3 siRNA transfection significantly reduced expression levels of GLUT3 mRNA and protein, leading to a strong growth inhibition and enhanced apoptosis ($P=0.017$) in AML cells. Moreover, treatment with GLUT3 siRNA, synergistically enhanced the cytotoxic and apoptotic effects of vincristine ($P=0.025$). We further investigated the possible mechanism involved in regulation of GLUT3 in AML cell proliferation and apoptosis. We found that GLUT3 negatively regulates EGFR activity, as well as the expression of its downstream proteins.

Conclusion: Our results demonstrated that GLUT3 plays a fundamental role in the survival and resistance of AML cells to vincristine. Therefore, GLUT3 can be considered as an attractive target for gene therapy of AML patients and siRNA-mediated silencing of this gene may be a novel strategy in AML treatment.

Keywords: Acute myeloid leukemia (AML), Chemosensitivity, Glucose transporter 3 (GLUT3), siRNA, Apoptosis

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Introduction

Acute myeloid leukemia (AML) consists of a group of clonal hematopoietic stem cell disorders characterized by failure to differentiate and ability to over-proliferate in the stem cell compartment leading to accumulation of myeloblasts.[1] Since identification of the BCR-ABL fusion gene, clinical treatment of this disease has changed from conventional hydroxyurea and busulfan treatment, which cause serious adverse events, to gene targeting drugs such as imatinib mesylate.[2] However, there are still enormous challenges in drug resistance induced by treatment.[3,4] Therefore, it's urgent to explore new therapies based on other mechanisms. As a regulator of early embryonic development, glucose transporter 3 (GLUT3) plays an important role in biological growth and development.[5] Meanwhile, GLUT3 is irreplaceable in development of the hematopoietic system.[6] Over-expression of GLUT3 can be found in most leukemia cells, suggesting that there are correlations between GLUT3 expression and the occurrence and development of leukemia.[7] This study was to investigate change in expression of the BCR-ABL fusion gene after GLUT3-RNAi silencing, and the effect of GLUT3 siRNA combined with chemotherapy on the proliferation and apoptosis of AML cells to provide new insights for clinical treatment of leukemia.

Materials and Methods

Cell Lines and Reagents

U937 and THP-1 leukemia cell lines were provided by the Biochemistry and Molecular Genetics Laboratory, Binzhou Medical University, and conventionally cultured. RPMI 1640 culture medium was purchased from Hyclone Co., Ltd, USA. Lipofectamine™ 2000 transfection reagent was purchased from Invitrogen (Cat#dx-3042, Carlsbad, CA, USA). GLUT3-siRNA and scrambled control siRNA (GTATGACAACAGCCTCAAG) were

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designed and synthesized by Shanghai GenePharma Co., Ltd. Total RNA extraction reagent, RNA reverse transcription kit and fluorescence quantitative PCR kit were purchased from TaKaRa company. Rabbit anti-human GLUT3 mAb (1:1000 dilution, Cat#3164,) and GAPDH mAb (1:1000 dilution, Cat#2845) were purchased from Santa Cruz Biotechnology Inc. Rabbit anti-human BCR-ABL mAb (Cat#3004) was purchased from Abcam company. Antibodies against EGFR, phosph-EGFR, ERK, phosph-ERK, JNK, phosph-JNK, p38, phosph-p38, PKC, phosph-PKC, GSK-3β, HCRP1, PTEN, and β-actin were purchased from Cell Signaling company. HRP-conjugated secondary antibodies were purchased from Hangzhou Xianzhi Biotechnology Co. Ltd. MTT reagent (Cat#sc-2004) was purchased from Sigma company. Annexin V-FITC Apoptosis Detection Kit was purchased from Jiangsu Keygen company. Vincristine (VCR) was purchased from Zhejiang Hisun pharmaceutical company.

qRT-PCR Analysis of GLUT3 mRNA Expression in Leukemia Cells After siRNA Transfection

U937 or THP-1 cells in the logarithmic growth phase were seeded in 6-well plates and transfected without or with GLUT3-siRNA or NC-siRNA using Lipofectamine™ 2000 reagent. Cells were collected after transfection for 24 hours according to the optimal transfection conditions. Total RNA was isolated using TRIzol reagent. According to TaKaRa reverse transcription kit, 1 µg total RNA was reversed into cDNA, and 2 µL cDNA product was added into 20 µL reaction system for real-time PCR to detect GLUT3 and BCR-ABL mRNA expression with following primers.
GLUT3 sense: 5'-GAACCTGTGCGAGTGGATG-3'; antisense: 5'-GGATGGTGATGTAGCGACTG-3'; BCR-ABL sense: 5'-CTTCTCCCTGGCATCCGTGGA-3'; antisense: 5'-TGCAACGAAAAGGTTGGGGT-3'; β-actin sense: 5'-CTCCATCCTGGCCTCGCTGT-3'; antisense: 5'-GCTGTCACCTTCACCGTTCC-3'. DNA was amplified by two-step method: 95°C, 35 seconds predegeneration; 95°C, 5 seconds, 58°C, 15 seconds, 40 cycles. mRNA expression was analyzed by $2^{-\Delta\Delta CT}$ method.

Western Blot Analysis of GLUT3 Protein Expression in AML Cells After siRNA Transfection

Cells in GLUT3-siRNA, NC-siRNA, and untransfection group were collected after transfection for 48 hours. Cells were lysed by the lysate on ice. Protein concentration was determined by the bicinchoninic acid (BCA) method. Fifty micrograms protein from each group was electrophoresed by SDS-PAGE electrophoresis and transferred to PVDF membrane. After blocking with 7% skim milk at room temperature for 2 hours, the membranes were incubated with primary antibodies overnight at 4°C. After rinsing the membranes to remove unbound antibodies, the membranes were incubated with HRP-conjugated secondary antibodies at room temperature for 2 hours. The membranes were washed by TBST and detected by the enhanced chemiluminescence (ECL) system. The relative expression of proteins was analyzed by chemiluminescence system (ECL, Pierce, Rockford, IL, USA). GAPDH or β-actin was used as internal control.

MTT Assay

The MTT assay was carried out to detect cell viability of leukemic cells with GLUT3 siRNA. The transfected cells (0.8×10^4) were seeded on 96-well plates and divided into 6 groups: the nontransfected group, the NC-siRNA group, the GLUT3-siRNA group, the VCR group, the NC-siRNA+VCR group, and the GLUT3-siRNA+VCR group (6 wells in each group). Cells were transfected with siRNA for 24 hours and 27 µg/µL VCR was added. Cells were cultured for 48 hours and then incubated with MTT solution (5 mg/mL, 20 µL) for 4 hours. After 4 hours, cells were centrifugated in dark and the supernatant was discarded, and 150 µL of DMSO was added to each well, vibrating 5 minutes in dark. The optical density (OD) value of samples at 490 nm was measured. Cell proliferation inhibition rate (%) is equal to (OD value in the normal cell group- OD value in the experimental group) divided by OD value in the normal cell group ×100%. The experiment was repeated 3 times.

Flow Cytometry

Cells were divided into 6 groups: the nontransfected group, the NC-siRNA group, the GLUT3-siRNA group, the NC-siRNA+VCR group, the VCR group, and the GLUT3-siRNA+VCR group (2 wells in each group). The U937 cells were transfected with siRNA for 24 hours and 25 µg/µL of VCR was added. Cells were collected after 24 hours post-transfection in each group and washed twice with cold PBS. Cells were resuspended in 500 µL binding buffer and incubated with 5 µL Annexin-V-FITC and 5 µL propidium iodide (PI) at room temperature for 15 minutes in the dark.

Statistical Analysis

All statistical analyses were performed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). All experiments were performed in triplicates and data are presented as mean ± SD. The statistical significance of differences between mean values was determined by the Tukey HSD test. The proliferation inhibitory rate of cells after GLUT3-siRNA transfection was compared using repeated measure analysis of variance. $P < 0.05$ was

considered to indicate a statistically significant difference.

Results

GLUT3 mRNA was downregulated in AML cells after GLUT3-siRNA transfection

Results from qRT-PCR analysis showed that the mRNA expression levels of GLUT3 and BCR-ABL were down-regulated in the GLUT3-siRNA group compared with those in the NC-siRNA group in AML cells (Figure 1A and B, $P < 0.05$). However, no obvious expression changes for GLUT3 or BCR-ABL mRNA were observed in the NC-siRNA group compared with the nontransfected group (Figure 1A and B, $P > 0.05$).

GLUT3 protein was downregulated in AML cells after GLUT3-siRNA transfection

Western blot results showed that the expression levels of GLUT3 protein and BCR-ABL protein were down-regulated in the GLUT3-siRNA group compared with the nontransfection group and the NC-siRNA group in U937 cells (Figure 2A). Similar results were also found in THP-1 cells (Figure 2B).

GLUT3-siRNA transfection combined with VCR inhibited AML cell proliferation

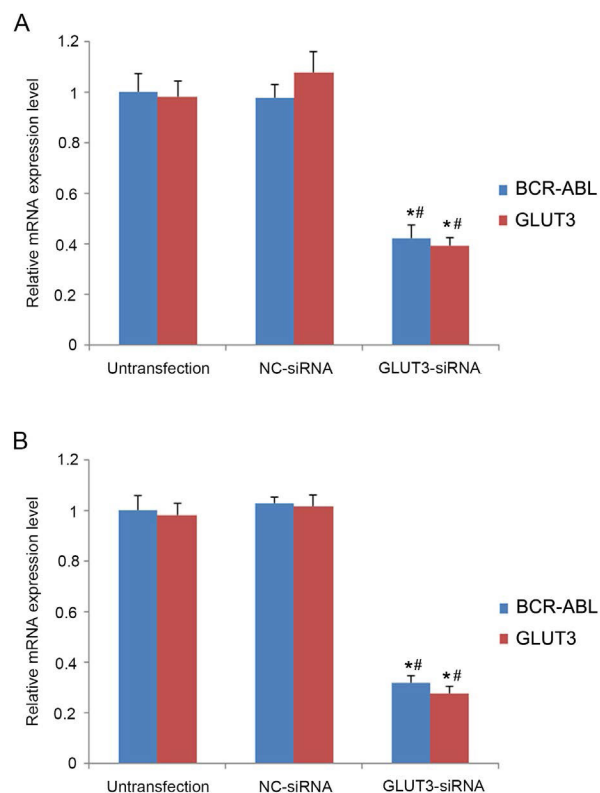The results of MTT showed that the cell proliferation inhibition rate in GLUT3-siRNA group was obviously higher than that of the nontransfected group and the NC-siRNA group (Figure 3A and B, $P = 0.023$). GLUT3-siRNA transfection combined with VCR could significantly inhibit cell proliferation, and the cell proliferation inhibition rate was higher than that of VCR
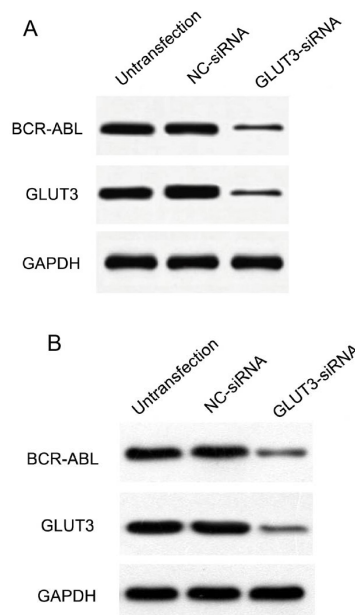

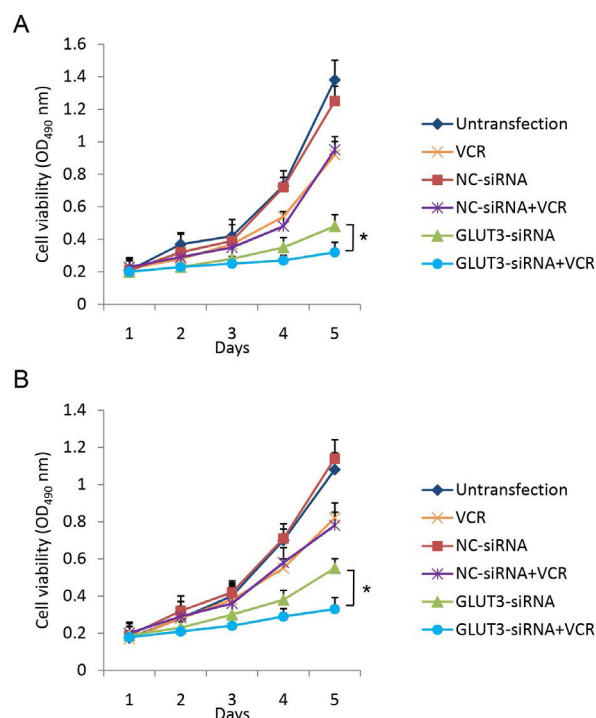**Figure 2.** Protein expression levels of GLUT3 and BCR-ABL in U937 **(A)** and THP-1 Cells **(B)** were detected by Western blot.

Figure 1. mRNA expression levels of GLUT3 and BCR-ABL were detected by qRT-PCR in U937 **(A)** and THP-1 cells **(B)**. *$P < 0.05$ vs. NC-siRNA group, # $P < 0.05$ vs. untransfection group.

Figure 3. Proliferation Inhibitory Rates of U937 **(A)** and THP-1 Cells **(B)** After GLUT3-siRNA Transfection. *$P < 0.05$.
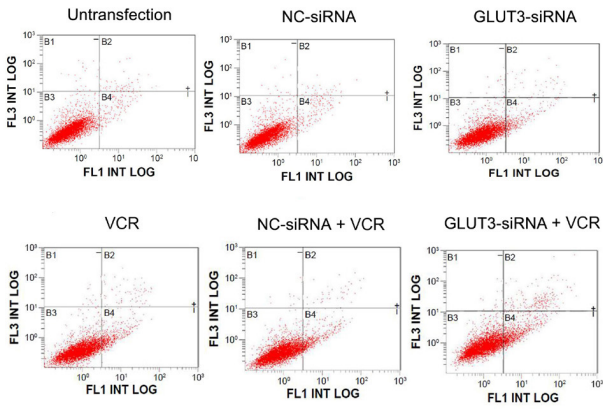
Figure 4. PGLUT3-siRNA transfection enhances VCR induced U937 cell apoptosis.

group and GLUT3-siRNA group in both U937 and THP-1 cells (P = 0.012) (Figure 3A and B, P < 0.05).

GLUT3-siRNA transfection combined with VCR promoted apoptosis of U937 cells

The results of flow cytometry showed that cell apoptosis rate in the GLUT3-siRNA + the VCR group was obviously higher than that of the VCR group and the GLUT3-siRNA group (Figure 4, P = 0.025). However, there was no significant difference in apoptosis rate between the VCR group and the NC-siRNA+VCR group (Figure 4).

GLUT3 inhibits EGFR activity and its downstream signaling pathways

We further investigated the possible mechanism by which GLUT3 inhibits proliferation and enhances apoptosis of U937 cells. We detected EGFR activity in leukemia cells and found that GLUT3 depletion upregulated p-EGFR expression (Figure 5). We next measured expression of downstream signaling molecules of EGFR. The MAPK-ERK signaling pathway has been well documented as

downstream of EGFR. Our results showed that GLUT3 depletion in U937 cells increased the phosphorylated form of MAPK family members. Moreover, activity of PKA and PKC was also increased. However, GSK-3β and PTEN, being negatively regulated by EGFR, were down-regulated (Figure 5). These findings collectively indicated that GLUT3 inhibits proliferation and enhances apoptosis via negatively regulating EGFR activation.

Discussion

GLUT3 is a glucose transporter that allows energy independent transport of glucose across the hydrophobic cell membrane, regulating its concentration. Pathological GLUT3 expression has been reported in various cancers such as gastric, testicular, ovarian, non-small cell lung, and breast cancer.[8-11] GLUT3 gene is closely related to renal cell carcinoma, and regarded as a tumor suppressor gene of intestinal tumors.[8] It has also been reported that inhibition of GLUT3 abolished survival advantage and suppressed the tumorigenic potential of liver tumor-initiating cells, indicating a role of GLUT3 in liver cancer.[13] Currently, more and more attention has been paid to the role of GLUT3 in the process of hematopoietic system development and the hematopoietic system pathology. It has been shown that the abnormal activation of GLUT3 in majority of leukemia cells can promote malignant transformation of hematopoietic cells.[9] Unfortunately, the tumorigenesis mechanism of GLUT3 depletion in the hematopoietic system is still not clear. In our study, the results showed that GLUT3-siRNA transfection reduced GLUT3 mRNA and protein expression significantly, while there was no significant change in the NC-siRNA group, suggesting that the designed and synthesized GLUT3-siRNA function was effective; therefore, the GLUT3-siRNA was used for further experiments. After GLUT3 gene silencing, BCR-ABL mRNA and protein expression were significantly decreased, while there was
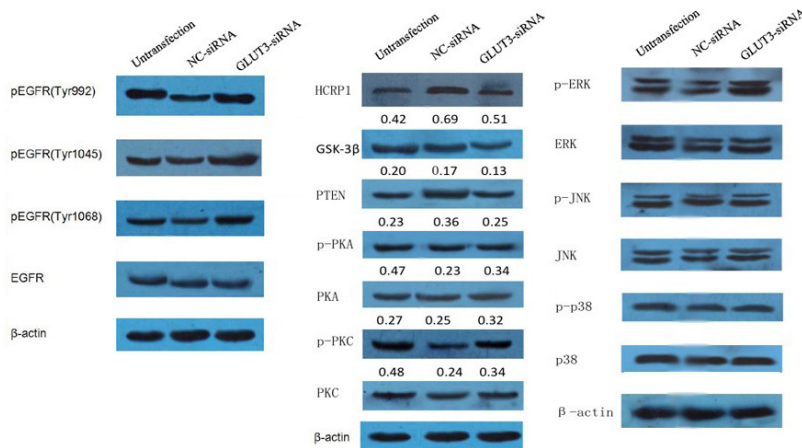

Figure 5. GLUT3 inhibits EGFR activity and downstream signaling proteins in U937 cells.

no obvious change in the NC-siRNA group, suggesting that GLUT3 gene silencing could down-regulate the expression of the BCR-ABL fusion gene.

GLUT3 is involved in multiple pathways, including cAMP, NF-kB, and p53 signaling.[15-17] EGFR plays a crucial role in tumor cell growth and apoptosis. EGFR belongs to a family of cell membrane receptor tyrosine kinases, which is phosphorylated and activated by its ligands, such as EGF, TGF-α, and heparin binding EGF. EGFR is degraded through the autophagy-lysosome pathway and related proteins are degraded by proteases.[10,11] Based on previous experiments, RNAi combined with VCR chemotherapy was used in U937 cells to determine their effect on proliferation and apoptosis of leukemia cells. Our results showed that AML cell proliferation was reduced and apoptosis was enhanced in the GLUT3-siRNA combined with the VCR group, compared with the nontransfected group and the VCR alone group, which suggests that GLUT3-siRNA combined with VCR can inhibit cell proliferation and promote cell apoptosis significantly, and GLUT3 silencing can enhance the sensitivity of AML cells to chemotherapeutic drugs.

Previous researches have confirmed that silencing expression of the fusion gene of BCR-ABL by RNAi can significantly inhibit proliferation and promote apoptosis in leukemia cells.[12,13] In this study, downregulation of BCR-ABL could be detected after GLUT3 gene silencing. On the other hand, some researchers found that GLUT3 silencing in gastric cancer cells may lead to increased expression of apoptosis factors such as PTEN and caspase-3.[14-16] Some reports found overexpression of PTEN and caspase-3 can inhibit intracellular drug efflux and increase sensitivity of tumors to chemotherapy drugs.[17,18] These reports indicated that GLUT3 siRNA combined with VCR can inhibit cell proliferation and promote cell apoptosis because GLUT3 depletion can downregulate BCR-ABL fusion gene expression, leading to increased caspase-3 and PTEN expression, which decrease intracellular VCR efflux, and thus enhance the sensitivity of leukemia cells to VCR.

In summary, in this study, GLUT3 siRNA was successfully constructed and effectively induced silencing of GLUT3 expression in leukemia cells. GLUT3-siRNA combined with VCR can inhibit cell proliferation and promote cell apoptosis significantly, suggesting that GLUT3 silencing can downregulate expression of the BCR-ABL fusion gene, which causes cell apoptosis and enhances the sensitivity of leukemia cells to VCR. GLUT3-siRNA combined with VCR can be new targets for treatment ideas in leukemia.

Authors' Contribution
Conception and intellectual input: ZY, BLX; design and performance of experimentation: ZY, ZJ; manuscript drafting: BLX. Statistical analyses and data interpretation: XX; All authors read and approved the final manuscript.

Conflict of Interest Disclosures
The authors have no conflicts of interest.

Ethical Statement
This study was carried out in strict accordance with the Institutional Animal Ethics Care and Use Committee of the First Affiliated Hospital of Wenzhou Medical University (approval No. 20450573). Informed consent was obtained from all individuals who participated in this study.

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