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Original Article

AMPK Activation in TET2 Downregulated Leukemia Cells Upon Glutamine Limitation

TET2 Baskılanmış Lösemi Hücrelerinde Glutamine Kısıtlamasında AMPK Aktivasyonu

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ABSTRACT

Objective: Metabolic rewiring is a characteristic of cancer cells. Cancer cells require more nutrients for survival and proliferation. Although glutamine can be produced in cells via a series of enzymatic reactions, a group of cancer cells are dependent on extracellular glutamine for survival. TET2 plays a role in DNA demethylation and is a tumor suppressor gene. The *TET2* gene is frequently mutated in various cancers, including acute myeloid leukemia (AML). Our study aimed to investigate the association between TET2-knockdown AML cell line HL-60 cells and glutamine metabolism.

Methods: To evaluate the association between TET2 expression and glutamine limitation, TET2 was downregulated in HL-60 cells using shRNA plasmids. The proliferation of TET2-knockdown HL-60 cells was calculated in normal and glutamine-deficient medium. GLUL mRNA expression was investigated using quantitative reverse transcription polymerase chain reaction and protein levels were evaluated using immunoblotting.

Results: The numbers and viability of TET2-knockdown HL-60 cells were decreased in low glutamine-containing medium, but the viability of TET2-knockdown HL-60 cells was higher than that of control cells. GLUL mRNA expressions were increased in TET2- knockdown cells in low glutamine. In addition, P-AMPKα protein expression was increased in TET2-knockdown HL-60 cells in low glutamine-containing medium.

Conclusions: Our findings indicate that TET2-knockdown HL-60 cells may be more resistant to glutamine deprivation. In glutamine-deficient medium, the mRNA expression of glutamine synthetase is increased, which could be related to glutamine addiction in cells. In addition, low-glutamyl medium increased the P-AMPKα protein level in TET2-knockdown HL-60 cells.

Keywords: Glutamine metabolism, TET2 expression, AML, AMPK, shRNA-mediated gene silencing

ÖZ

Amaç: Metabolik yeniden programlama, kanser hücrelerinin ayırt edici bir özelliğidir. Kanser hücreleri, hayatta kalmak ve çoğalmak için tümör mikroçevresinden glukoz ve glutamin alımını artırır. Glutamin *de novo* sentezlenebilmesine rağmen, birçok kanser hücresi hayatta kalabilmek için hücre dışı glutamine bağımlıdır. DNA demetilasyonunda görev alan *TET2* geni, aynı zamanda bir tümör baskılayıcı gen olarak bilinir. *TET2* geni, akut miyeloid lösemi (AML) dahil olmak üzere çeşitli kanserlerde sıklıkla mutasyona uğrar. Çalışmamız, *TET2* geni baskılanmış AML hücre hattı HL-60 hücreleri ile glutamin metabolizması arasındaki ilişkiyi araştırmayı amaçladı.

Yöntemler: TET2 ekspresyonu ile glutamin sınırlaması arasındaki ilişkiyi değerlendirmek için *TET2* geni, shRNA plazmitleri kullanılarak HL-60 hücrelerinde baskılandı. TET2-baskılanmış HL-60 hücrelerinin hücre proliferasyonu, normal ve düşük glutamin ortamındaki hücre sayıları ile hesaplandı. mRNA ekspresyonlarındaki değişiklikler, kantitatif ters transkriptaz kullanılarak araştırıldı. GLUL, AMPK-α ve P-AMPKα protein ekspresyonu immünoblotlama ile değerlendirildi.

Bulgular: Düşük glutamin ortamında TET2-baskılanmış HL-60 hücrelerinin hücre sayıları ve hücre canlılığı azaldı. TET2-baskılanmış HL-60 hücrelerinin hücre canlılığının kontrol hücrelerinden daha yüksek olduğu bulundu. Düşük glutaminde TET2-baskılanmış hücrelerde GLUL mRNA ekspresyonunun arttığı bulundu. Ayrıca, düşük glutamin ortamında TET2- baskılanmış HL-60 hücrelerinde P-AMPKα protein ekspresyonunun arttığı bulundu.

Sonuçlar: Bulgularımız, TET2-baskılanmış HL-60 hücrelerinin, glutamin yoksunluğuna karşı daha dirençli olabileceğini ve düşük glutamin ortamının, HL-60 hücrelerinin glutamin bağımlılığı ile bağlantılı olarak, glutamin metabolizmasında belirli genlerin ekspresyonunu artırdığını göstermektedir. Ek olarak, düşük glutamin ortamı, TET2-baskılanmış HL-60 hücrelerinde P-AMPKα protein seviyesini artırdı.

Anahtar kelimeler: Glutamin metabolizması, TET2 ekspresyonu, AML, AMPK, shRNA aracılı gen susturma

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INTRODUCTION

Cancer cells constantly modify their metabolism due to the increased need for nutrients for survival, and hematologic malignancies are not the exception of this phenomenon[1]. Acute myeloid leukemia (AML), a common type of acute leukemia in adults, is caused by abnormal proliferation and differentiation of hematopoietic progenitor cells[2].

To maintain energy homeostasis, 5'-adenosine monophosphate (AMP)-activated protein kinase (AMPK) controls ATP production and consumption[3]. AMPK is a serine/threonine kinase family member and a heterotrimeric protein complex consisting of three subunits, α, β- and γ-subunits. At low cellular energy levels, AMPK is activated by the phosphorylation of threonine 172 (Thr172) in the kinase domain of the α-subunit[3-5]. AMPK functions by phosphorylating downstream targets to suppress ATP-consuming pathways and increase ATP-producing pathways[6]. In addition to maintaining energy homeostasis, AMPK plays significant roles in tumorigenesis by regulating cellular growth, autophagy, inflammation, stress responses, and cell polarity[5,7].

Glutamine is crucial for nitrogen (in nucleotides and amino acid biosynthesis) and carbon (in lipid and ATP biosynthesis) sources and is thus highly demanded by rapidly proliferating cells[8-10]. Glutamine synthetase (GS), encoded by GLUL, synthesizes *de novo* glutamine from purines and pyrimidines in a highly controlled process[11]. Despite this process, a group of cancer cells have been shown to be addicted to extracellular glutamine[1,8,12].

TET2 is a member of the ten-eleven translocation protein family (TET1-3) and is a well-known tumor suppressor[13]. TET2, an Fe (II)- and α-ketoglutarate (α-KG)-dependent dioxygenase, modulates active DNA demethylation by oxidizing 5-methylcytosine (5-mC), which is methylated by DNA methyltransferase, to 5-hydroxymethylcytosine (5-hmC)[14,15]. The loss of TET2 function due to mutations is associated with DNA hypermethylation and therefore transcriptional reprograming that promotes leukemogenesis[16-18].

This study aimed to investigate the association between TET2 expression and glutamine metabolism in HL-60 cells. For this aim, an shRNA-mediated gene silencing method was applied to downregulate TET2 in the HL-60 cell line, and then cell proliferation, expression levels of the GLUL gene involved in glutamine metabolism, and AMPK activity were determined in glutamine-deficient medium.

MATERIALS and METHODS

Cell Culture

HL-60 cells were cultured in RPMI 1640 (Gibco, Thermo Fisher, USA) supplemented with 10% fetal bovine serum, 1% l-glutamine, and a mixture of 1% penicillin-streptomycin in a 5% CO_2-humidified atmosphere at 37 °C.

Glutamine Limitation

HL-60 cells were cultured in RPMI 1640 medium without glucose/glutamine (Cat. No. P04-17550, PAN-Biotech, Germany) supplemented with 10% FBS, 10 mM glucose, and 1% pen/strep. The amount of glutamine in the FBS is 50 mM. Glutamine was added to its conventional glutamine counterpart at a final concentration of 2 mM. Cells with/without glutamine were cultivated for 3 days. For cell counting, manual counting was performed using a hemocytometer, and the percentage of cell proliferation in glutamine-limited compared with the normal medium was calculated.

Downregulation of TET2 Expression

Two puromycin-resistant shRNA plasmids targeting TET2 were purchased from Qiagen to knockdown TET2 (Sure-Silencing shRNA plasmids, Cat No KH17943P). TET2-targeting shRNAs cloned in puromycin-resistant plasmids were transformed into DH5a-competent bacteria. Subsequently, HL-60 cells were transfected according to the shRNA plasmid manufacturer's protocol. Wild-type HL-60 cells were used as the control group. 0.5 µg plasmid was used for transfection. Puromycin selection was applied for 3 days, and the medium was replaced. quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed to confirm downregulation of TET2 expression.

CellTiter Cell Viability Assay

CellTiter-Glo® Luminescent Cell Viability Assay (Promega, USA) was used for ATP measurement. HL-60 control and TET2-downregulated cells were seeded at a concentration of 2500 cells/well and incubated for 96 h. After incubation, 40 µL of reagent was added to each well, and the mixture was mixed for 5 min on an orbital shaker. The luminescence values were measured using a Cytation 5 Cell Imaging Multi-Mode Reader (BioTek, USA).

Quantitative Reverse Transcription Polymerase Chain Reaction

Single-stranded cDNA was synthesized from total RNA using a High-Capacity RNA-to-cDNA Synthesis Kit

Table 1. The primers sequences.	
Gene	**Primer sequence (5'-3')**
TET2	F: TGTTAGAAAGGAGACCCGACTG R: TTCCATTCTGGAGCTTTGTAGC
GLUL	F: TGGGAACTGGAATGGTGC R: CGTTGATGTTGGAGGTTTCATG
RPLP0	F: AGCATCTACAACCCTGAAGTG R: AGCAAGTGGGAAGGTGTAATC

(Applied Biosystems, USA). SYBR Green PCR Master Mix was used for the qRT-PCR reactions on the RotorGene (Qiagen) instrument. The primers listed in Table 1 were used to analyze TET2 and GLUL mRNA expression. The relative gene expression level was determined by comparing with RPLP0. Fold changes in mRNA levels were calculated using ΔΔCt method.

Western Blotting

After 72 h of culture with/without glutamine medium, TET2-downregulated HL-60 and control HL-60 cell lines were harvested, then washed with 1× PBS and lysed. BCA Protein Assay Kit (Pierce, Thermo Fisher, USA) was used to determine protein concentrations and 10 µg of proteins were electrophoresed in a 12% polyacrylamide gel. AMPKα, P-AMPKα and β-actin primary antibodies with HRP-conjugated secondary antibodies from Cell Signaling Technology (Beverly, MA) were used for blotting. The membranes visualized with an enhanced chemiluminescence substrate (SuperSignal, Thermo Fisher, USA) using the Azure C300 gel imaging system. Quantification of the bands in the blots was performed using the ImageJ software.

Statistical Analysis

The results are expressed as means ± standard deviation. Student's t-test with Welch's correction was used for the statistical analysis of the comparisons of data. Statistical analysis and graphing were performed using GraphPad V9 (GraphPad Software, San Diego, CA, USA). All experiments were performed in triplicate except for the viability experiment with quintuple technical replicates.

RESULTS

Glutamine Limitation Increases TET2 mRNA Expression in HL-60 Cells

We evaluated TET2 expression in response to glutamine limitation. It was found that in 50 µM glutamine-containing medium, TET2 mRNA expression was nearly doubled compared with that in 2 mM glutamine-containing normal medium (*p<0.05) (Figure 1).

Downregulation of TET2

As TET2 mRNA expression was found to increase in glutamine-deficient medium (50 µM glutamine), we downregulated its expression. TET2 mRNA expression was downregulated by approximately 50% compared with the control plasmid using two different shRNAs (**p<0.01, ***p<0.001) (Figure 2).

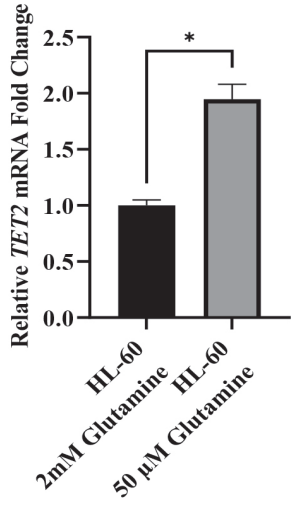

Figure 1. Effect of glutamine limitation on TET2 mRNA expression. TET2 mRNA expression was increased by nearly 2-fold in low glutamine-containing medium in HL-60 cells. Data are presented as mean ± SD, *p<0.05.

SD: Standard deviation

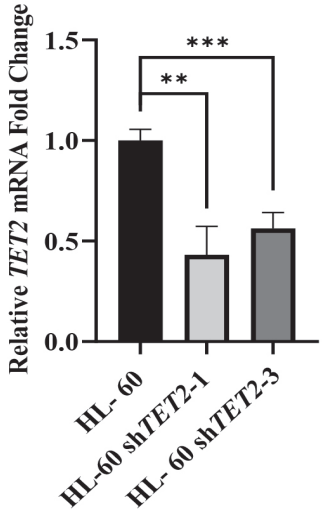

Figure 2. shRNA-mediated downregulation of TET2 mRNA expression. Two different shRNA decreased mRNA expression to half that of control HL-60 cells. Data presented as mean ± SD, **p<0.01, ***p<0.001.

SD: Standard deviation

Cell Proliferation in Glutamine-deficient Medium

In comparison with the control group, total cell number declined in HL-60 shTET2-1 cells in 50 μM glutamine (*p<0.05), whereas it was slightly increased in HL-60 sh*TET2*-3 cells, which was not statistically significant (^{ns}p≥0.05) (Figure 3).

Cell Viability in Low-glutamine Medium

In glutamine-deficient medium, the survival percentage decreased from 100% to 52.79% in the control group, to 69.81% in HL-60 shTET2-1 cells, and to 70.53% in HL-60 shTET2-3 cells (shTET2-1; *p<0.05, and shTET2-3; **p<0.01). Additionally, we found that cell viability decreased in the glutamine-deficient condition compared with the normal concentration (Figure 4).

GLUL mRNA and Protein Expression in TET2-knockdown HL-60 Cells Under Low Glutamine

GLUL mRNA expression was increased in all cells in glutamine-deficient medium (Figure 5A). Although the increase in low glutamine levels in HL-60 shTET2-1 cells was statistically significant (*p<0.05), the increase in HL-60 and HL-60 shTET2-3 cells was not statistically significant (^{ns}p≥0.05). Moreover, GS protein expression was increased in all cell lines under glutamine-deficient conditions (Figure 5B). Additionally, GS protein expression in TET2-knockdown HL-60 cells was remotely increased in cells incubated with low glutamine compared with control cells, which was not statistically significant (^{ns}p≥0.05) (Figure 5C).

AMPK-α and Phospho-AMPK-α Protein Expressions in TET2-knockdown HL-60 Cells Under Low Glutamine Condition

Immunoblotting was used to assess the regulation of AMPK-α and P-AMPK-α protein expressions in the low glutamine medium. To confirm increased activation of AMPK-α, oligomycin complex (Cayman Chemical, USA) was added to HL-60 cells at a ratio of 1:1000 for 3 days in normal and low glutamine medium. AMPK-α protein expression was examined to control AMPK activity in the HL-60 cell line (Figures 6A, B).

P-AMPK-α protein expression was increased at low glutamine levels in all three cell lines, although statistically significant only in HL-60 shTET2-3 cells (Figures 6A, C). P-AMPK-α protein expression in TET2-knockdown HL-60 cells was increased compared with

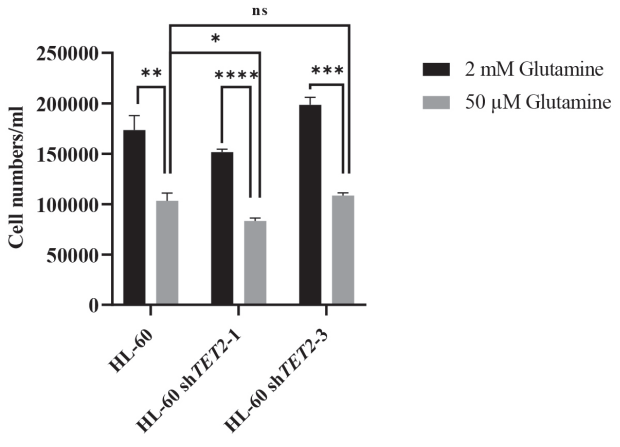

Figure 3. Cell proliferation in normal (2 mM) and low-glutamine (50 μM) medium. Cell proliferation was calculated using cell numbers. Cells were plated at a concentration of 20,000 cells/mL and cultured for 72 h. Then, cells were washed and counted using a hemacytometer. Cell numbers decreased at low glutamine concentrations in all cell lines. In the presence of low glutamine, HL-60 shTET2-1 cell counts and HL-60 shTET2-3 cell counts increased. Data are presented as mean ± SD. nsp≥0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

SD: Standard deviation

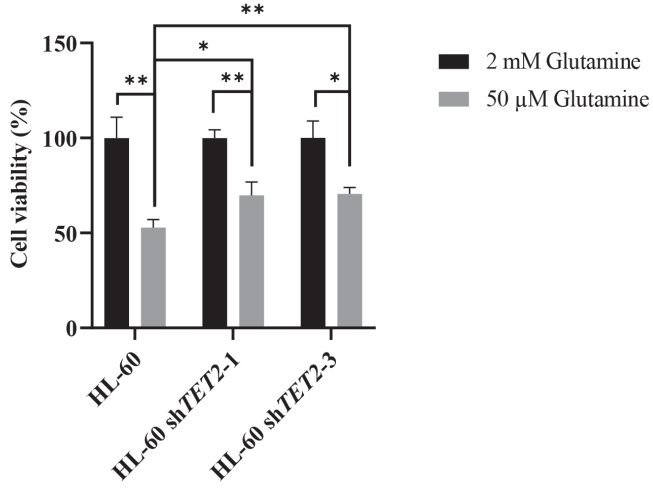

Figure 4. Effect of low glutamine medium on cell viability in TET2-downregulated HL-60 cells. Cells were plated at a concentration of 20,000 cells/mL and cultured for 4 days. Cell viability was assessed using the CellTiter-Glo® Luminescent Cell Viability Assay. shRNA1 and shRNA2 OD values compared with control cells in percentage. Cell viability was found to be higher in TET2-downregulated HL-60 cells in low glutamine-containing medium compared to control cells. Data are presented as mean ± SD, *p<0.05, **p<0.01.

SD: Standard deviation

the control cell line in both normal and low glutamine media. In addition, we found that P-AMPK-α expression increased in TET2-knockdown cells under low glutamine compared to normal glutamine.

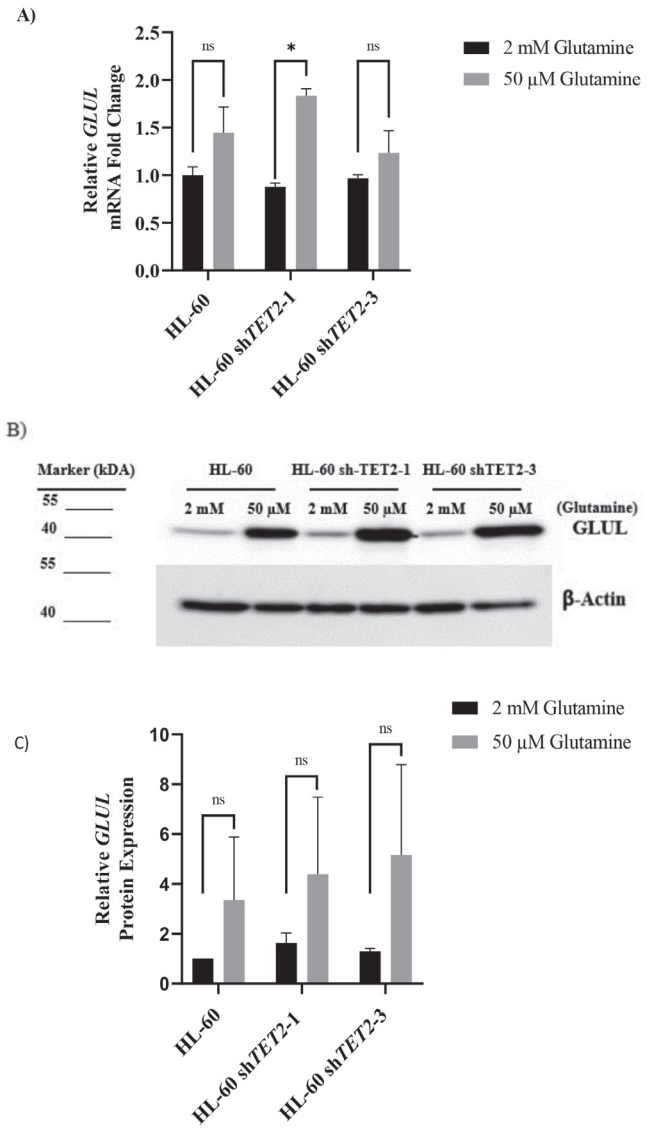

Figure 5. Glutamine synthetase (GLUL) mRNA expression and protein levels of TET2-knockdown HL-60 cells in low glutamine medium. **A)** GS-encoding relative GLUL mRNA expression levels in 2 mM and 50 µM glutamine-containing medium. **B)** Western blot analysis and **C)** Quantification of GLUL protein expression were performed in TET2-knockdown and control HL-60 cells cultured in medium containing 2 mM and 50 µM glutamine. β-actin was used as a housekeeping protein. Data are presented as mean ± SD, $^{ns}p \geq 0.05$, $p < 0.05$, $^{**}p < 0.01$.

SD: Standard deviation

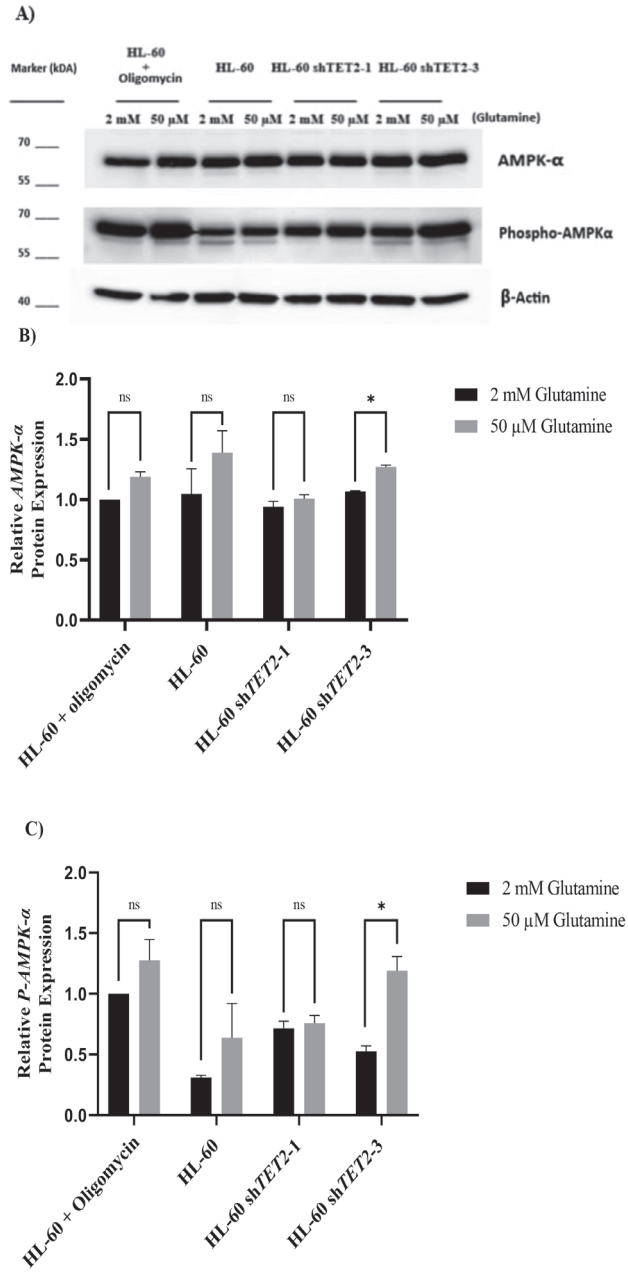

Figure 6. A) Immunoblotting and quantification of **B)** AMPK-α and **C)** Phospho-AMPK-α proteins were performed in TET2-knockdown and control HL-60 cells cultured in medium containing 2 mM and 50 µM glutamine. Control cells were treated with oligomycin to confirm the increased activation of AMPK-α and P-AMPK-α. P-AMPK-α protein expression was increased in TET2-knockdown cells in low glutamine compared with normal glutamine levels, as well as in control cells. α-actin was used as a housekeeping protein. Data are presented as mean ± SD, $^{ns}p \geq 0.05$.

SD: Standard deviation

DISCUSSION

Glutamine plays an important role in ATP synthesis as well as most carbon and nitrogen metabolism for cancer cell proliferation[9]. Low glutamine levels have been observed in tumor microenvironments *in vivo* and *in vitro* in various types of cancer. Therefore, cancer cells adapt their metabolism to their environment for survival and proliferation[12,19]. Glutamine deprivation in many cancer cell types, including AML, has been shown to cause cell death through glutamine addiction[20].

A study with 4 different AML cell lines showed that the cell line most addicted to glutamine was HL-60 cells, and the proliferation of HL-60 cells was reduced by glutamine restriction and rescued by the addition of the tricarboxylic acid cycle intermediate oxaloacetic acid[21]. It was shown that a group of AML cell lines become addicted to glutamine for cell proliferation, and the level of glucose in the tumor microenvironment does not affect this addiction[12]. In addition to glutamine deprivation, inhibition of glutamine metabolism by the glutaminase inhibitor CB-839 reduced antioxidant glutathione production, leading to mitochondrial ROS accumulation and apoptotic cell death in several AML cell lines[22].

The *TET2* gene encodes a member of the TET family of enzymes that alter the epigenetic state of DNA by converting 5-mC to 5-hmC. Somatic loss-of-function mutations in TET2 are associated with poor prognosis and advanced disease progression in myelodysplastic syndromes, AML, and chronic myelomonocytic leukemia[17,23]. Loss of TET2 function models have shown that TET2 plays a role in the regulation of myeloid progenitor cell proliferation and differentiation[24].

This study found an almost 2-fold increase in TET2 mRNA expression in HL-60 cells in low glutamine medium (Figure 1). TET2 expression was reduced in HL-60 cells using two different shRNA-mediated plasmids (Figure 2), and cell proliferation and viability were assessed under normal and low glutamine conditions. Cell counts were decreased in both control and TET2-downregulated cells in glutamine-deficient medium (Figure 3). In both normal and low glutamine media, HL-60 shTET2-1 cells showed less proliferation than control cells. In contrast, the opposite result was observed in HL-60 shTET2-3 cells. Furthermore, the viability of TET2-downregulated HL-60 cells in low glutamine-containing medium was higher than that of control HL-60 cells. Our results suggest that downregulation of TET2 expression in HL-60 cells is resistant to glutamine deficiency.

In a recent study, TET2 expression in the K562 cell line was downregulated by the shRNA-mediated system, and it was found that downregulation of TET2 did not change cell proliferation, but TET2-downregulated K562 cells were more resistant to CAPE treatment[25]. In a study of SUM149 triple-negative breast cancer cells, it was shown that metabolically adaptable SUM149-MA cells obtained by culturing SUM149 cells in a medium lacking glutamine had a 90% lower TET2 protein level and selected an undifferentiated therapy-resistant phenotype similar to that of TET2-mutant cancer[26].

Under normoxic conditions (20% O_2), the HepG2 human hepatoma cell line was found to increase TET2 and TET3 mRNA levels when cultured with low glucose (5 mM) or glutamine (0.5 mM). In hypoxic conditions (1% O_2), the mRNA levels of *TET* genes decreased more in the presence of low glucose than in the presence of low glutamine[27].

The GS enzyme synthesizes glutamate from glutamate and ammonia in an ATP-dependent manner[28]. High GLUL expression is linked to poor prognosis in liver cancer, ovarian cancer, glioblastoma, and hepatocellular carcinoma[12,29,30]. In addition, when the *GLUL* gene was knocked out in the HL-60 cell line, it was found that GLUL-knockout cells were shown to have decreased cell viability in both glucose- and glutamine-deficient media[12].

Our results showed that in low glutamine-containing media, GLUL mRNA expression was elevated in both TET2-knockdown HL-60 cells and control cells. However, the change was significant only in HL60 shTET2-1 cells (Figure 5A). The differences in GLUL mRNA expression levels between HL-60 shTET2-1 and HL-60 shTET2-3 cells were attributed to variations in the rate of TET2 expression knockdown. Furthermore, our immunoblotting results confirmed the qPCR results, showing that the GLUL protein level was increased in all cells in low glutamine-containing medium, and the increase in GLUL expression was higher in TET2-knockdown cells (Figure 5B).

It has been shown that AMPK alters DNA methylation by phosphorylating TET2 and plays an important role in cell differentiation[31,32]. Recently, AMPK was shown to phosphorylate TET2 at serine 99. However, hyperglycemia-mediated AMPK inactivation was found to result in the inhibition of AMPK-mediated TET2 phosphorylation and increased calpain-mediated degradation. Treatment with metformin increased 5-hmC levels and suppressed tumor growth by maintaining AMPK-mediated TET2 phosphorylation, indicating the tumor suppressor role

of TET2[31]. Furthermore, a study of monocytic U937 cells showed that it activates AMPK and adaptive mechanisms to overcome glutamine deficiency in response to glutamine starvation[33].

In endometrial carcinoma, silencing of *AMPK* gene expression using siRNA has been found to significantly decrease TET2 expression and 5-hmC levels, and metformin treatment regulates TET2 expression by activating AMPK. In addition, siRNA-mediated TET2 knockdown increased the proliferation of cancer cells[34]. In human AML U937 cells, exposure to malignant progression-inducing hydroquinone (HQ) increased AMPK activity, resulting in increased TET2 and FOXP3 expression in both U937 and U937/HQ cells[35].

We determined higher P-AMPK-α protein levels in TET2-downregulated HL-60 cells than in control cells in low glutamine-containing medium (Figure 6). Therefore, we speculate that the knockdown of *TET2* gene expression in HL-60 cells may increase the energy demand of cells in low glutamine-containing media. In the literature, it has been shown that TET2 expression is regulated by glucose-dependent AMPK activity[31]. Our results suggest that, in addition to the literature, a low-glutamine medium may regulate TET2 expression by activating AMPK.

CONCLUSION

Our findings indicate that the knockdown of *TET2* gene expression in HL-60 cells treated with shRNA reduced cell viability and proliferation in low glutamine-containing media. Furthermore, cell viability in TET2-downregulated cells was higher in low glutamine concentrations than in control HL-60 cells. We also found that GLUL expression and P-AMPK-α protein levels were increased in TET2-downregulated HL-60 cells in low glutamine concentrations. In further studies, TET2 expression can be completely silenced by the *CRISPR/Cas9* gene editing method, and the mRNA expression and protein levels of genes related to glutamine metabolism, such as GLUL, GLS1, GLS2, and GLUD1, can also be examined. To further investigate the association between TET2 and AMPK, it is necessary to determine which TET2 residue is phosphorylated by AMPK in low-glucose media. In TET2-downregulated HL-60 cells, 5-hmC levels should be assessed in normal and low-glucose media.

Ethics

Ethics Committee Approval: Our research is conducted only with cell lines *in vitro* conditions. Thus, no ethical approval is required.

Informed Consent: No informed consent is required.

Author Contributions

Design: B.Y., Data Collection and/or Processing: A.M.B., Analysis and/or Interpretation: A.M.B., B.Y., Literature Search: A.M.B., B.Y., Writing: A.M.B., B.Y.

Conflict of Interest: The authors have no conflict of interest to declare.

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