PTEN/AKT signaling mediates chemoresistance in refractory acute myeloid leukemia through enhanced glycolysis

MIN JEONG RYU[1,2], JEONGSU HAN[1,3], SOO JEONG KIM[1,3], MIN JOUNG LEE[1-4], XIANSHU JU[1,3,4], YU LIM LEE[1,3,4], JEONG HWAN SON[4], JIANCHEN CUI[1,3,4], YUNSEON JANG[1,3,4], WOOSUK CHUNG[5,6], IK-CHAN SONG[7], GI RYANG KWEON[1,2,4] and JUN YOUNG HEO[1,3-5]

[1]Department of Biochemistry, College of Medicine; [2]Research Institute for Medical Science; [3]Infection Control Convergence Research Center; [4]Department of Medical Science; [5]Brain Research Institute, Chungnam National University School of Medicine; Departments of [6]Anesthesiology and Pain Medicine, and [7]Internal Medicine, Chungnam National University Hospital, Daejeon 35015, Republic of Korea

Received January 21, 2019; Accepted July 9, 2019

DOI: 10.3892/or.2019.7308

Abstract. Primary refractory acute myeloid leukemia (AML) and early recurrence of leukemic cells are among the most difficult hurdles to overcome in the treatment of AML. Moreover, uncertainties surrounding the molecular mechanism underlying refractory AML pose a challenge when it comes to developing novel therapeutic drugs. However, accumulating evidence suggests a contribution of phosphatase and tensin homolog (PTEN)/protein kinase B (AKT) signaling to the development of refractory AML. To assess PTEN/AKT signaling in AML, two types of AML cell lines were evaluated, namely control HL60 cells and KG1α cells, a refractory AML cell line that is resistant to idarubicin and cytarabine (AraC) treatment. Changes in the expression level of glycolysis- and mitochondrial oxidative phosphorylation-related genes and proteins were evaluated by reverse transcription-quantitative polymerase chain reaction and western blot analyses, respectively. The mitochondrial oxygen consumption and extracellular acidification rates were measured using an XF24 analyzer. CCK8 assay and Annexin V/PI staining were used to analyze cell viability and cellular apoptosis, respectively. The PTEN protein was found to be depleted, whereas AKT phosphorylation levels were elevated in KG1α cells compared with HL60 cells. These changes were associated with increased expression of glucose transporter 1 and hexokinase 2, and increased lactate production. AKT inhibition decreased the proliferation of KG1α cells and decreased extracellular acidification without affecting HL60 cells. Notably, AKT inhibition increased the susceptibility of KG1α cells to chemotherapy with idarubicin and AraC. Taken together, the findings of the present study indicate that activation of AKT by PTEN deficiency sustains the refractory AML status through enhancement of glycolysis and mitochondrial respiration, effects that may be rescued by inhibiting AKT activity.

Correspondence to: Dr Jun Young Heo or Professor Gi Ryang Kweon, Department of Biochemistry, College of Medicine, Chungnam National University School of Medicine, Munhwa-dong, Jungu, Daejeon 35015, Republic of Korea
E-mail: junyoung3@gmail.com
E-mail: mitochondria@cnu.ac.kr

Abbreviations: R/R AML, relapsed/refractory acute myeloid leukemia; LSC, leukemia stem cell; ECAR, extracellular acidification rate; OCR, oxygen consumption rate; RT-qPCR, reverse transcription-quantitative polymerase chain reaction

Key words: refractory acute myeloid leukemia, chemoresistance, phosphatase and tensin homolog, protein kinase B, glycolysis

Introduction

Acute myeloid leukemia (AML) is characterized by proliferation of immature myeloid leukocytes in the bone marrow as a consequence of genetic insults, such as mutations (1,2). To improve the probability of remission in AML patients, clinical researchers have employed a dose-intensification strategy using combined chemotherapy with idarubicin or daunorubicin and cytarabine (AraC) (3,4). Although such efforts have been shown to increase overall survival, up to 30% of adults and 70% of elderly individuals (>65 years old) with AML fail to achieve complete remission after induction chemotherapy, a condition termed relapsed/refractory (R/R) AML (5,6).

Efforts have been made to develop novel, targeted drugs for uncontrolled R/R AML, but such efforts are hampered by our lack of understanding of the pathogenesis and molecular mechanism underlying R/R AML. Intensive combination chemotherapy with high-dose AraC, with or without gemtuzumab ozogamicin, has been shown to achieve a complete remission rate of only ~30% in R/R AML patients (7). Recently, fms-related tyrosine kinase 3 (FLT3) containing an internal tandem duplication (FLT3-ITD) has been identified as a prognostic molecular marker in patients with a high recurrence rate and poor outcome, and is considered a treatment target for R/R AML patients (8). The pan-kinase inhibitor midostaurin, a staurosporine derivative, has recently

been approved by the US Food and Drug Administration for patients with FLT3-mutant AML, but the variability of genetic mutations in AML makes it difficult to reduce the recurrence rate of R/R AML by targeting specific genes (9,10).

A leukemia stem cell (LSC)-positive population of cells that is dependent on oxidative respiration rather than glycolysis for energy generation has been suggested to be a major cause of R/R AML (11). Notably, a recent report demonstrated that tigecycline, which targets mitochondrial translation, selectively kills leukemic stem and progenitor cells *in vivo* in AML and chronic myeloid leukemia (CML) (12,13). However, the association of genes that regulate intracellular signaling pathways and mitochondrial oxidative phosphorylation/glycolysis with AML drug resistance is poorly understood at present.

Phosphatase and tensin homolog (PTEN) is a phosphatase of the lipid signaling intermediate, phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which regulates phosphoinositide 3-phosphate (PI3K) and protein kinase B (AKT) signaling. The PI3K/AKT pathway is known to promote the survival and growth of human solid tumors and hematological malignancies in response to extracellular signals, such as growth factors, hormones and cytokines (14-16). Moreover, loss of PTEN activity through mutations, deletions or promoter methylation is frequently found in solid tumors and hematological malignancies (17-19). Although mutations in the PTEN gene are rarely observed in AML patients (~1 in 59 patients) (20), PTEN expression is generally downregulated in these patients (20). Moreover, PTEN is considered to be a biomarker in elderly patients with R/R AML (21). PTEN is crucial for the proliferation and differentiation of hematopoietic stem cells (HSCs), and PTEN-null mice initially develop a myeloproliferative disorder, followed by leukemia (22,23). In addition, PTEN acts as a tumor suppressor in LSCs without damaging normal HSCs or compromising hematopoiesis (24). However, whether PTEN mutations are associated with AML cell proliferation and changes in metabolic flow during the refractory period remains unknown.

PTEN-null mouse embryonic fibroblasts exhibit metabolic changes in association with downregulation of pyruvate kinase 2, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) and glutaminase, consistent with an anti-Warburg effect (25). In addition, mitochondrial mass is increased and glycolysis is induced in PTEN-null hepatocytes via estrogen-related receptor α (26). The present study, using these reports as a takeoff point, focused on the involvement of PTEN as a stemness gene in leukemic cell proliferation and induction of metabolic flow changes in the context of R/R AML. KG1α refractory AML cells and HL60 wild-type control cells were used to investigate the association between PTEN/AKT signaling and metabolic shifts in R/R AML.

Materials and methods

Chemicals, reagents and antibodies. Oligomycin (O4876), carbonyl cyanide m-chlorophenyl hydrazone (CCCP; C2759), rotenone (R8875), and AKT inhibitor (A6730) were purchased from Sigma-Aldrich; Merck KGaA. TRIzol was purchased from Invitrogen; Thermo Fisher Scientific, Inc. The Cell Counting Kit-8 (CCK8) was purchased from Dojindo Molecular Technologies, Inc. Anti-actin (rabbit polyclonal), anti-PARP (rabbit polyclonal), anti-GAPDH (rabbit polyclonal) and anti-hexokinase (HK)2 (rabbit polyclonal) antibodies were purchased from Santa Cruz Biotechnology, Inc. Antibodies against p-AKT (Thr308) (rabbit polyclonal), total AKT (rabbit polyclonal) and caspase-3 (rabbit polyclonal) were from Cell Signaling Technology, Inc. Antibody against oxidative phosphorylation I subunit, NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 8 (anti-NDUFB8) was obtained from Invitrogen; Thermo Fisher Scientific, Inc., and antibody against NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 9 (anti-NDUFA9) was purchased from Abcam.

Human leukemia cell lines. HL60 (ATCC® CCL-240™) and KG1α (ATCC® CCL-246.1™) cells were purchased from American Type Culture Collection and cultured in Iscove's Modified Dulbecco's Medium (Welgene, Inc.) supplemented with 20% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified 5% CO_2 atmosphere. The two cell lines were authenticated by STR genotyping. Plasmocin™ Prophylactic (InvivoGen) was used as a routine addition in liquid media to prevent mycoplasma contamination in cell cultures. All cell lines were used in our laboratory for <3 months after resuscitation.

Cell proliferation and cytotoxicity measurement. For proliferation rate assays, HL60 and KG1α cells in complete medium were counted and seeded in 96-well culture plates at $2x10^3$ cells per well. For measurement of drug cytotoxicity, cells were seeded at 100% confluence in 96-well cell culture plates and treated with drugs for 24 h. Thereafter, 10 μl of CCK8 reagent was added to each well and the plates were incubated for an additional 2 h. Absorbance was then measured at 450 nm using a Multiskan Ascent microplate spectrophotometer (Thermo Fisher Scientific, Inc.). To quantify apoptosis, necrosis and late apoptosis, cells were stained using an FITC Annexin V Apoptosis Detection Kit (BD Bioscience). After seeding at 100% confluence in 96-well cell culture plates and treating with drugs for 24 h, the cells were washed with 1 ml PBS and resuspended in 100 μl staining buffer containing 2 μl of Annexin V-FITC and 2 μl propidium iodide (PI). The cells were then incubated for 15 min at room temperature, followed by addition of 400 μl of staining buffer and quantification of fluorescence by flow cytometry. Fluorescence in Annexin V-FITC (505-560 nm) and PI (595-642 nm) channels was measured, and at least 10,000 single-cell events per sample were collected. Using a gating strategy dependent on the fluorescence intensity of Annexin V-FITC and PI, cell populations were divided into double-negative (live) cells, Annexin V-positive (apoptotic) cells, and double-positive (late apoptotic or necroptotic) cells (27).

Western blot analysis. Whole-cell lysates from HL60 and KG1α cells were prepared using RIPA lysis buffer [100 mM Tris-HCl pH 8.5, 200 mM NaCl, 5 mM EDTA, 0.2% sodium dodecyl sulfate (SDS)] containing a phosphatase and protease inhibitor cocktail (INtRON BioTechnology). Equal amounts of protein (20 μg), quantified by the Bradford assay (Bio-Rad Laboratories, Inc.), were resolved by SDS-PAGE (polyacrylamide 6 or 12% gel electrophoresis) and transferred

to an Amersham Protran nitrocellulose membrane (pore size, 0.2 μm; Amersham; GE Healthcare Life Sciences). The membranes were blocked by incubating for 1 h with TBST (10 mM Tris-HCL pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 3% skimmed milk, and were then sequentially incubated with primary antibody (16 h at 4˚C) and the appropriate horseradish peroxidase-conjugated secondary antibody (2 h at room temperature). Specific antibody-labeled proteins were detected using an enhanced chemiluminescence (ECL) system (WEST-ZOL plus; INtRON BioTechnology).

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). OCR and ECAR were measured using an *xf24* analyzer (Seahorse Bioscience). On day-1, the *xf24* biosensor cartridge was pre-incubated with 1 ml of *xf24* calibration buffer and then incubated at 37˚C overnight without CO_2. The cells were acutely treated as described with AKT inhibitor, idarubicin and/or AraC, delivered via port A, for 24 h. For measurement of OCR, HL60 and KG1α cells were seeded onto *xf24* cell culture microplates at $1x10^4$ cells per well in 0.5 ml *xf24* medium consisting of Dulbecco's modified Eagle's medium (pH 7.4) supplemented with 10 mM glucose, 1 mM sodium pyruvate and 2 mM L-glutamine (without sodium bicarbonate), and then incubated at 37˚C without CO_2 for 1 h. The *xf24* analyzer was operated according to the manufacturer's basic protocol at 37˚C with real-time injection of drugs. After equilibrating for 20 min, each well of the *xf24* cartridge was sequentially injected with the ATPase inhibitor oligomycin (2 μg/ml), the uncoupling agent CCCP (5 μM) and the mitochondrial electron transport inhibitor rotenone (2 μM), and OCR and ECAR were measured in real time. For glycolysis stress tests, HL60 and KG1α cells ($1x10^4$ cells per well) were seeded onto *xf24* cell culture microplates containing 0.5 ml Agilent Seahorse XF Base Medium (Seahorse Bioscience) and 1 mM L-glutamine, and then incubated at 37˚C without CO_2 for 45 min. Each well of the *xf24* cartridge was sequentially injected with glucose (10 mM) for glycolysis, oligomycin (1 μM) for glycolytic capacity and 2-deoxy glucose for glycolytic reserve measurement.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. cDNA was synthesized from 20 μg of total RNA using M-MLV Reverse Transcriptase and oligo-dT primers (Invitrogen; Thermo Fisher Scientific, Inc.). RT-qPCR was performed using cDNA, SYBR Green PCR Master Mix (iCycler iQ Real-Time PCR Detection System; Bio-Rad Laboratories, Inc.) and the following primer pairs: HK1, 5'-ggc cac gat gta gtc acc tt-3' (forward) and 5'-cac gtc cag gtc aaa ttc ct-3' (reverse); HK2, 5'-cca cct ttg tga ggt cca ct-3' (forward) and 5'-gtc ctc agg gat ggc ata ga -3' (reverse); SLC2A1, 5'-ccc aga tct ttg gtc tgg aa-3' (forward) and 5'-gac ttt cag ggc aaa atg ga-3' (reverse); PFK1, 5'-aga gcg tttc gat gat gct t-3' (forward) and 5'-gtt gta ggc agc tcg gag tc-3' (reverse); PDK3, 5'-cta ggt ggt ggt gtc cca ct-3' (forward) and 5'-taa cca aat cca gcc aaa gg-3' (reverse); and 18s ribosomal RNA, 5'-ctg gtt gat cct gcc agt ag-3' (forward) and 5'-cga cca aag gaa cca taa ct-3' (reverse). The relative expression of target mRNAs was quantified and normalized with respect to that of 18S rRNA, which was used as an endogenous control. Rotor-Gene 6000 real-time rotary analyzer software (version 1.7; Corbett Life Science-Qiagen) was utilized along with the $2^{-\Delta\Delta Cq}$ method (28).

Statistical analysis. Results are presented as mean ± standard error of the mean from at least three independent experiments. A two-tailed unpaired Student's t-test or one-way analysis of variance with Friedman test were performed using Graph Pad InStat software (GraphPad Software, Inc.). A P-value <0.05 was considered to indicate statistically significant differences.

Results

Downregulation of PTEN and phosphorylated AKT is associated with refractory AML. LSCs contribute to the central pathogenesis of R/R AML by sustaining stemness properties and continued propagation of leukemic cells after induction therapy. Using the KG1α leukemia cell line as an LSC-like cell model (29) with potential relevance to refractory AML (30), we first investigated responsiveness to the AML chemotherapeutic agents, idarubicin and AraC, compared with HL60 cells. Interestingly, treatment with idarubicin or AraC alone differentially affected cell viability, causing ~30% less cytotoxicity against KG1α cells compared with HL60 cells. Notably, combined treatment with idarubicin and AraC for 24 h also decreased the viability of HL60 cells without affecting KG1α cells (Fig. 1A). These changes in cell viability induced by idarubicin and AraC treatment were associated with diminished induction of cleaved poly(ADP-ribose) polymerase (PARP) and cleaved caspase-3 in KG1α cells, indicating that the remaining KG1α cells are resistant to and less damaged by idarubicin and AraC (Fig. 1B). To determine the death rate in HL60 cells treated with idarubicin, AraC and idarubicin plus AraC, Annexin V/PI staining was performed with quantification by flow cytometry. This analysis revealed that idarubicin, AraC and idarubicin/AraC increased Annexin V staining in HL60 cells (CTL, 0.5%; idarubicin, 66.3%; AraC, 1.3%; idarubicin/AraC, 63.86%), which is indicative of early apoptosis. Moreover, idarubicin, AraC and idarubicin/AraC increased the percentage of Annexin V/PI double-stained HL60 cells (CTL, 0.6%; idarubicin, 12.5%; AraC, 8.7%; idarubicin/AraC, 31.6%), which is indicative of late apoptosis and necroptosis (Fig. 1C-E). However, KG1α cells exhibited a slight increase in the ratio of Annexin V/PI double-stained cells by idarubicin/AraC. These results suggest that idarubicin and AraC were associated with a higher apoptotic ratio in HL60 cells compared with KG1α cells. Next, in order to determine the differences in PTEN expression in refractory AML, PTEN protein expression was assessed in KG1α and HL60 cells, and PTEN protein levels were found to be downregulated in KG1α cells compared with HL60 cells (Fig. 1F). Given previous reports suggesting that Thr308 phosphorylation of AKT is correlated with poor overall survival of AML patients (31), the AKT phosphorylation status was confirmed in the two cell lines. AKT was found to be constitutively phosphorylated at Thr308 in KG1α cells (i.e., in the absence of a stimulus) (Fig. 1F). Collectively, these findings indicate that low expression of PTEN and high expression/phosphorylation of

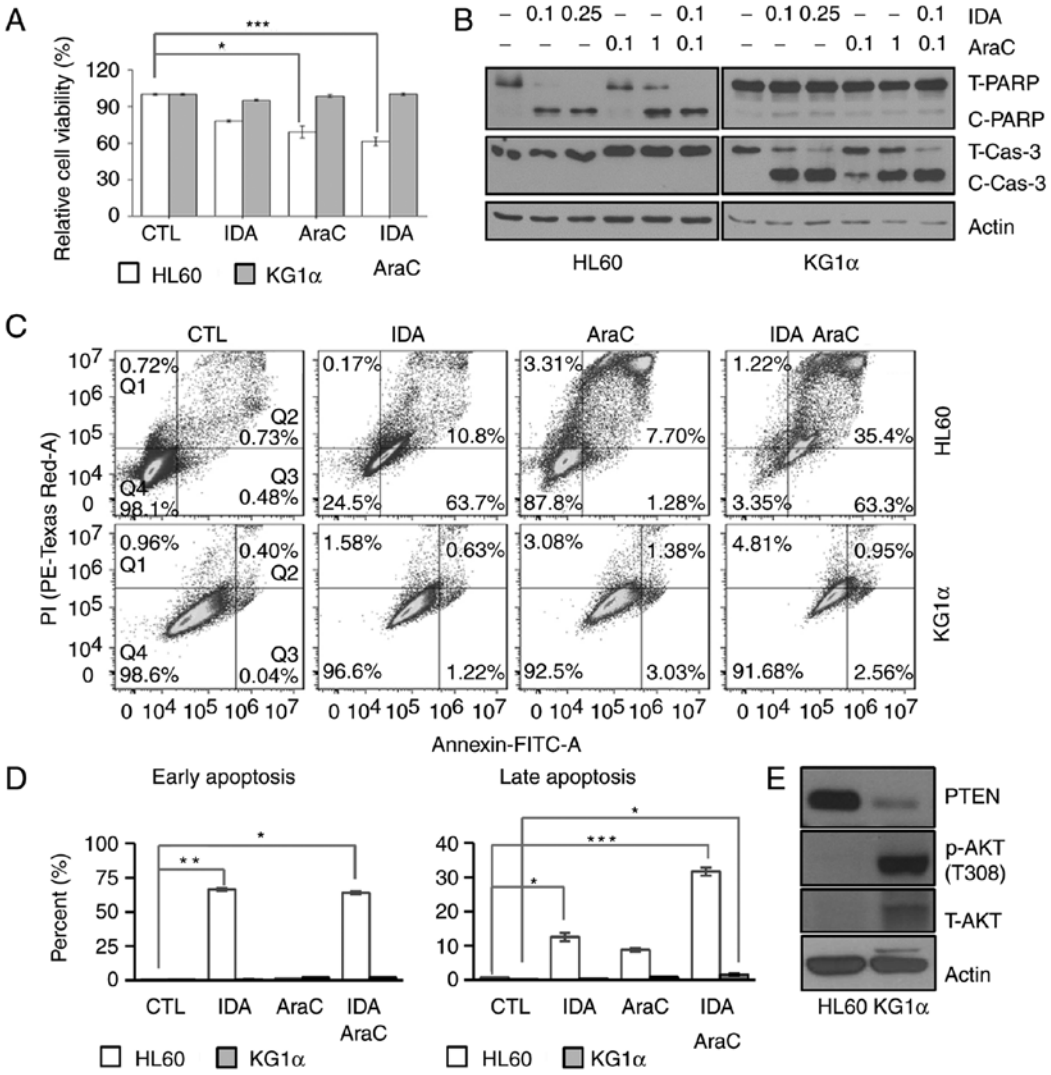

Figure 1. Differences in drug sensitivity and PTEN/AKT levels between HL60 and KG1α cells. (A) The viability of HL60 and KG1α cells, with/without treatment with 0.1 and 0.25 μM idarubicin and 0.1 and 1 μg/ml AraC, was measured by CCK8 assays (n=8, *P<0.05, ***P<0.005 vs. CTL). (B) Western blot analysis of the apoptosis marker proteins PARP and cleaved caspase-3; actin was used as an internal control. (C and D) Dot plot for flow cytometric analysis of apoptotic cells after Annexin V-FITC/PI staining in HL60 and KG1α cells treated with 0.1 μM idarubicin and 0.1 μg/ml AraC. Living cells (Q4) tested negative for both Annexin V-FITC and PI. Populations testing Annexin V-positive/PI-negative were classified as early-stage apoptotic cells (Q3), and double-positive cells were classified as late-stage apoptotic cells (Q2). (E) Percentage of early and late apoptotic HL60 and KG1α cells following idarubicin and AraC treatment. (n=3, *P<0.05, **P<0.01, ***P<0.005 vs. CTL). (F) Western blot analysis of PTEN, p-AKT (Thr308) and total AKT in HL60 and KG1α cells. PTEN, phosphatase and tensin homologue; AKT, protein kinase B; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; AraC, cytarabine; IDA, idarubicin; CTL, control.

AKT are correlated with a refractory AML phenotype against combined treatment with idarubicin and AraC.

The levels of glycolytic metabolism-related enzymes are higher in KG1α cells compared with HL60 cells. AKT is involved in the regulation of cell proliferation and death. Notably, it was recently demonstrated that overexpression of a constitutively active AKT mutant in cancer cells upregulates glycolysis by inducing glucose transporter (GLUT)4 and HK, thereby leading to sustained growth and enhanced cancer cell survival (32). Moreover, activation of the AKT cascade effectively induced GLUT1 gene expression in mouse hepatoma Hepa1c1c7 cells (33). Accordingly, as AKT levels were higher in refractory AML KG1α cells compared with HL60 cells, we assessed the expression of genes involved in regulating glucose uptake and metabolic flow, including mitochondrial enzymes, by RT-qPCR and western blotting. First, we found that the mRNA expression levels of GLUT1/SLC2A1 were increased in KG1α cells compared with HL60 cells (Fig. 2A). Moreover, HK1 and HK2, the initial enzymes of glycolysis, were expressed at higher levels in KG1α compared with HL60 cells (Fig. 2B and C). In addition, the expression of phosphofructokinase 1, the most important regulatory enzyme in this process, was increased more than two-fold in KG1α compared with HL60 cells. (Fig. 2D). Consistent with these mRNA expression data, the GLUT1 and HK2 proteins were also more highly expressed in KG1α compared with HL60 cells (Fig. 2F). The final product of glycolysis is pyruvate, which is translocated to the mitochondria for the generation of mitochondrial complex I and II substrates via the tricarboxylic acid cycle. The pyruvate dehydrogenase complex converts pyruvate to acetyl CoA, which is downregulated by pyruvate dehydro-

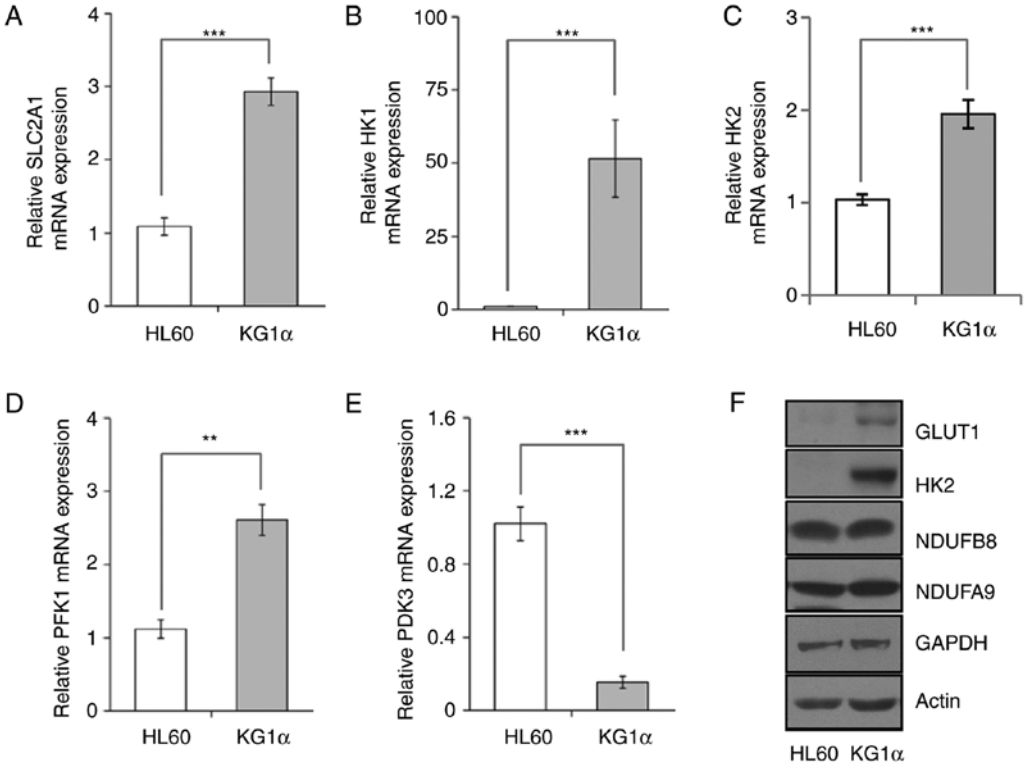

Figure 2. Expression of glycolysis-related genes in HL60 and KG1α cells. (A-E) mRNA levels of (A) SLC2A1/GLUT1, (B) HK1, (C) HK2, (D) PFK1 and (E) PDK3, as determined by quantitative polymerase chain reaction analysis (n=5 or 6, **P<0.01, ***P<0.005 vs. HL60). (F) Western blot analysis of GLUT1, HK2, NDUFB8, NDUFA9 and GAPDH; actin was used as a loading control. GLUT, glucose transporter; HK, hexokinase; PFK, phosphofructokinase; PDK, pyruvate dehydrogenase kinase.

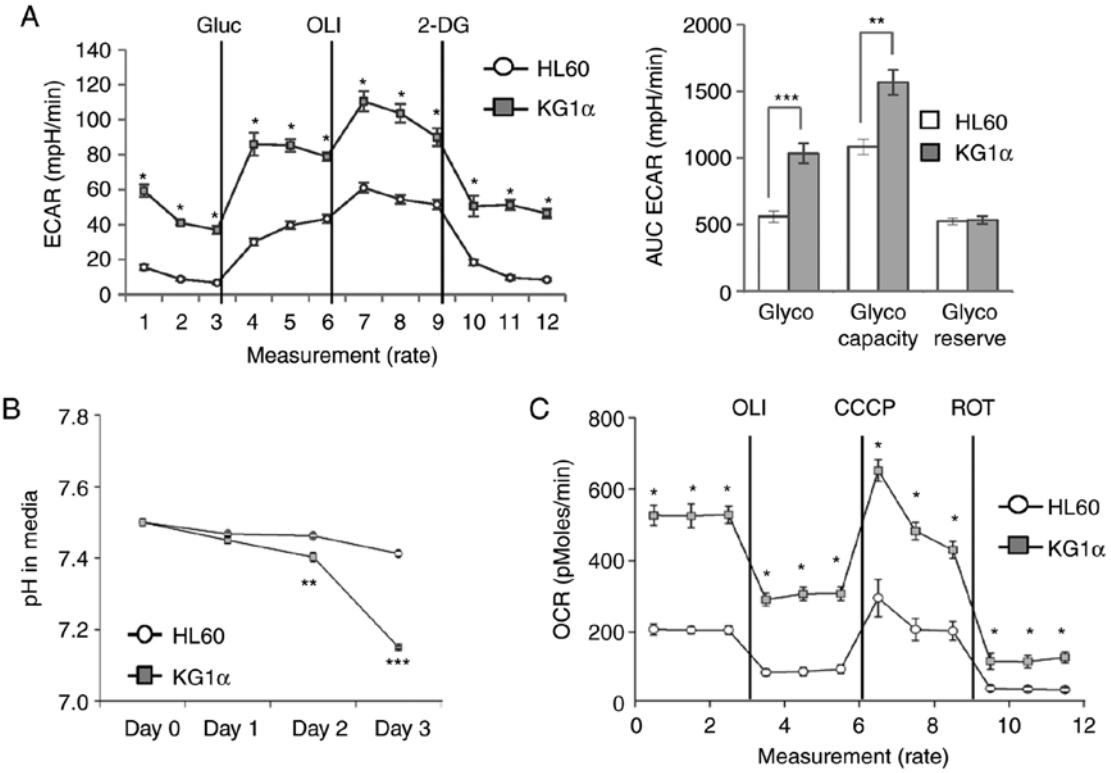

Figure 3. Metabolic phenotype in HL60 and KG1α cells. (A) ECAR in HL60 and KG1α cells, determined using an *xf24* analyzer. Glucose (Gluc; 10 mM) was used for glycolysis; oligomycin (OLI; 1 μM) was used for glycolytic capacity; and 2-deoxyglucose (2-DG; 50 mM) was used for calculation of the glycolytic reserve. Left panel, real-time ECAR; right panel, quantification of each step of ECAR (n=4, *P<0.05, **P<0.01, ***P<0.005 vs. HL60). (B) pH levels in media after culturing HL60 and KG1α cells for 48 h (n=4, **P<0.01, ***P<0.005 vs. HL60). (C) OCR in HL60 and KG1α cells, determined using an *xf24* analyzer (n=7 or 8, *P<0.05 vs. HL60). OLI 2 μg/ml was used for uncoupling; CCCP (5 μM) was used to induce maximal OCR; and rotenone (ROT, 2 μM) was used to assess non-mitochondrial OCR. ECAR, extracellular acidification rate; OCR, oxygen consumption rate.

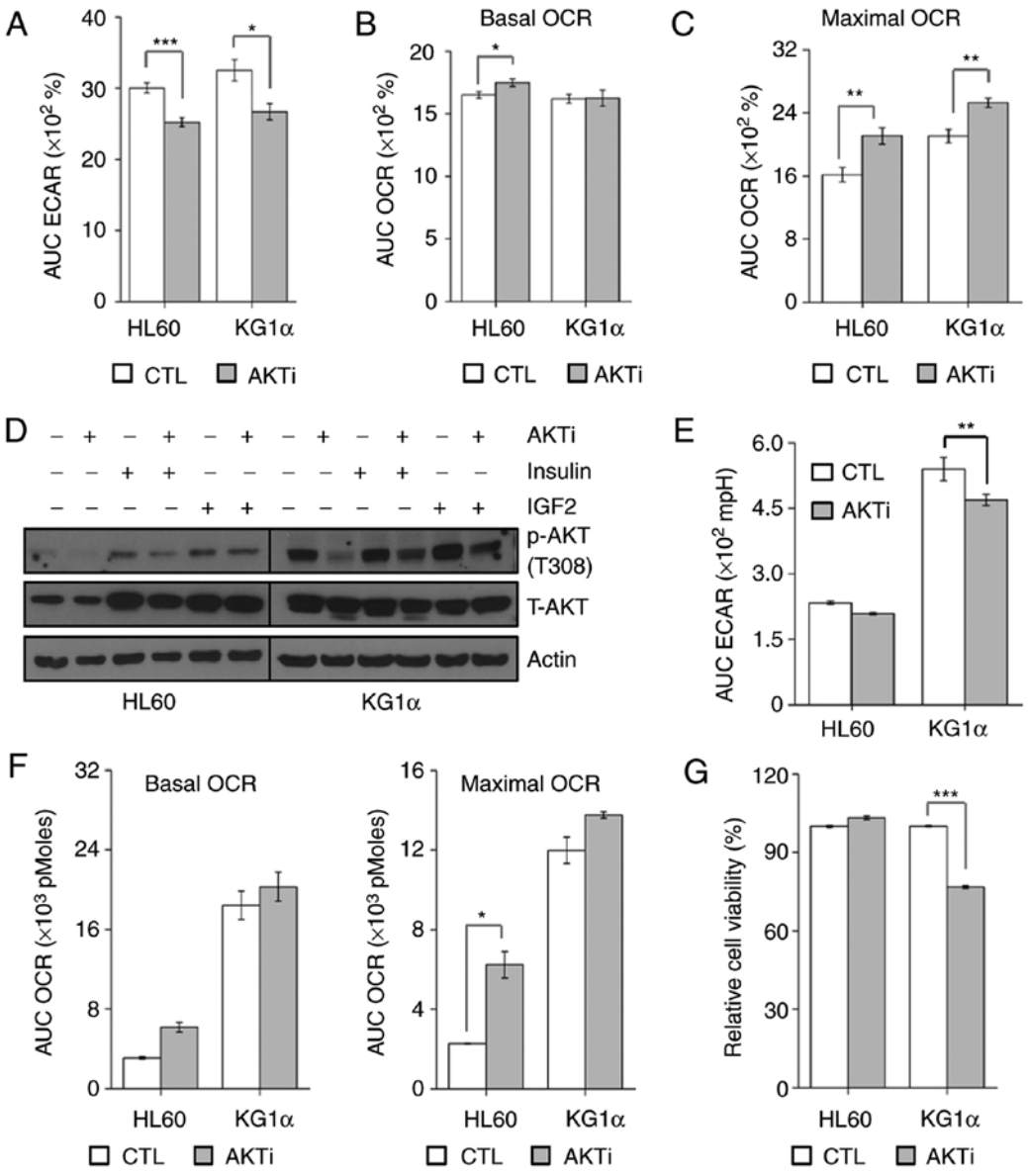

Figure 4. AKT inhibition reduces the proliferation of KG1α cells. (A and B) Measurement of (A) ECAR and (B) OCR in KG1α cells treated with AKT inhibitor (AKTi) for 20 min (n=4, *P<0.05, ***P<0.005 vs. CTL). (C) CCCP (5 μM) induces maximal OCR in KG1α cells treated with AKTi for 40 min (n=4, **P<0.001 vs. CTL). (D) Immunoblotting of total cell lysates for p-AKT (Thr308) and total AKT after treatment with AKTi (1 μM) for 6 h with/without insulin (100 nM) or IGF2 (100 ng/ml) for 2 h. Actin was used as a loading control. (E) Measurement of ECAR in HL60 and KG1α cells treated with AKTi (n=4, **P<0.001 vs. CTL). (F) Measurement of OCR in HL60 and KG1α cells treated with AKTi (n=4, *P<0.05 vs. CTL). CCCP (5 μM) was used to induce maximal OCR. (G) Viability of HL60 and KG1α cells after treatment with 1 μM AKTi for 24 h, determined by CCK8 assay (n=8, ***P<0.005 vs. CTL). ECAR, extracellular acidification rate; OCR, oxygen consumption rate; CTL, control; IGF, insulin-like growth factor.

genase kinase (PDK) in the mitochondria. As expected, the PDK3 expression levels were lower in KG1α cells compared with HL60 cells, thereby enhancing mitochondrial respiration by supplying the substrates, NADH and FADH, despite the absence of a change in the expression levels of mitochondrial complex subunit proteins (Fig. 2E).

Mitochondrial respiration and glycolysis capacity are higher in KG1α compared with HL60 cells. To verify the correlation between upregulated expression of glycolytic genes and physiological changes in glycolysis rate in KG1α cells, glycolytic activity was assessed by measuring the ECAR and OCR using an *xf24* analyzer. Consistent with the observed gene expression profile, a glucose stress test using glucose, oligomycin and 2-deoxyglucose showed higher glycolytic capacity in KG1α cells compared with that in HL60 cells (Fig. 3A). Next, cellular acid production was quantified by measuring the hydrogen concentration in the medium. Compared with HL60 cells, KG1α cells maintained a low-pH environment from day 2 onwards (Fig. 3B). Interestingly, basal and maximal OCRs were also higher in KG1α cells compared with those in HL 60 cells (Fig. 3C). Taken together, these data indicate that PTEN-mutant KG1α cells are characterized by more prominent mitochondrial respiration and glycolytic pathway flux compared with HL60 cells.

Inhibition of AKT inhibits refractory AML cell growth through downregulation of glycolysis. To confirm the role

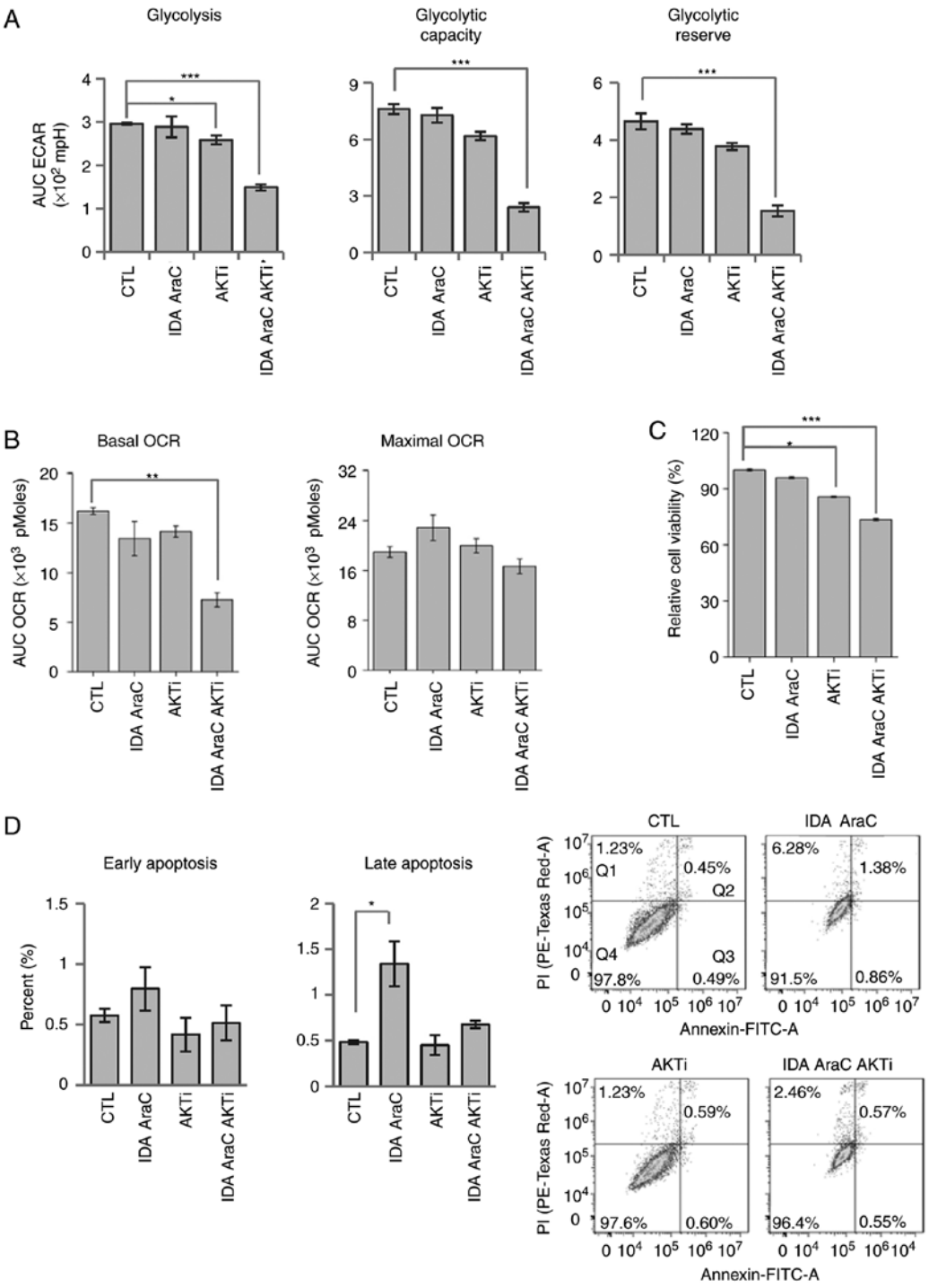

Figure 5. AKT inhibition reduces the viability of chemoresistant KG1α cells. (A) Measurement of ECAR in KG1α cells treated with IDA, AraC, and AKT inhibitor (AKTi) for 24 h. Quantification of glycolysis, glycolytic capacity, and glycolytic reserve. (n=4, *P<0.05, ***P<0.005 vs. CTL). (B) Measurement of OCR in KG1α cells treated with IDA, AraC, and AKTi for 24 h (n=4, **P<0.01 vs. CTL). CCCP (5 μM) was used to induce maximal OCR. (C) Viability of KG1α cells treated with IDA, AraC and AKTi for 24 h, as determined by CCK8 assays. (n=8, *P<0.05, ***P<0.005 vs. CTL). (D) Percentage of early and late apoptotic KG1α cells following treatment with IDA, AraC and AKTi for 24 h (n=3, *P<0.05 vs. CTL). Living cells (Q4) tested negative for both Annexin V-FITC and PI. Populations testing Annexin V-positive/PI-negative were classed as early-stage apoptotic cells (Q3), whereas double-positive cells were classed as late-stage apoptotic cells (Q2). ECAR, extracellular acidification rate; OCR, oxygen consumption rate; AraC, cytarabine; IDA, idarubicin; PI, propidium iodide; CTL, control.

of AKT in refractory AML cell proliferation, mitochondrial OCR and ECAR were measured in HL60 and KG1α cells in real time using an *xf24* analyzer following introduction of an AKT inhibitor into an *xf24* cartridge. As expected, acute injection of the AKT inhibitor reduced ECAR and enhanced maximal and basal OCR (Fig. 4A-C). Due to the low levels of AKT phosphorylation in HL60 cells, the effect of AKT inhibitors on AKT phosphorylation was confirmed by basal or pharmacological stimulation. Since it is known that insulin and insulin growth factor (IGF)2 promote AKT phosphorylation and affect cell survival in AML (34,35), we investigated AKT phosphorylation following treatment with

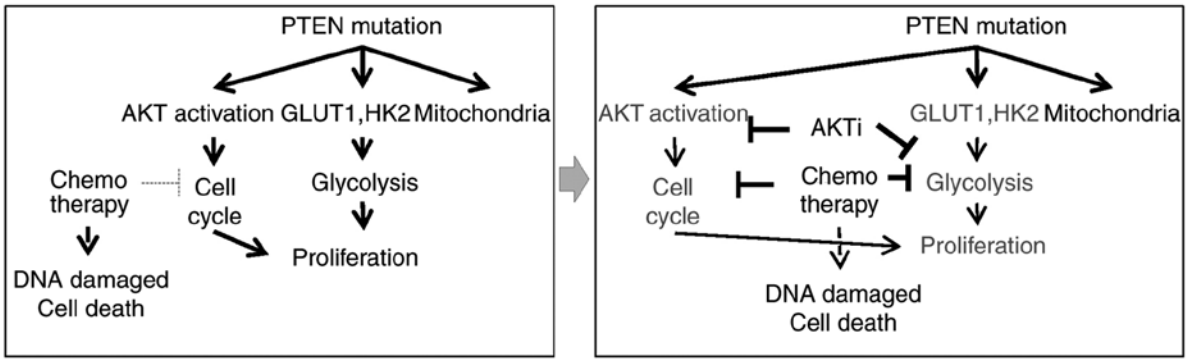

Figure 6. Model describing the effects of AKT inhibitors on chemotherapy in PTEN-mutant, refractory AML cells. PTEN, phosphatase and tensin homologue; AML, acute myeloid leukemia; GLUT, glucose transporter; HK, hexokinase.

insulin and IGF2 in HL60 and KG1α cells. Pretreatment with AKT inhibitor was applied 4 h prior to the addition of 100 nM insulin, IGF2 (100 ng/ml) or vehicle for 2 h. As a result, AKT inhibitors reduced AKT phosphorylation under basal and pharmacological-stimulated conditions in HL60 and KG1α cells (Fig. 4D). Under these conditions, ECAR was prolonged and decreased in KG1α cells, but OCR remained unchanged (Fig. 4E and F). Inhibition of AKT ultimately reduced cell proliferation only in AKT-hypersensitive KG1α cells (Fig. 4G). These results suggest that AKT activation affects refractory AML cell proliferation in a glycolysis-dependent manner.

Targeting the PTEN/AKT pathway by inhibiting AKT enhances the drug sensitivity of refractory AML cells. As noted above (see Fig. 1A), refractory AML KG1α cells exhibited diminished responsiveness to chemotherapy (idarubicin and AraC). To test the role of AKT in drug sensitivity, KG1α cells were treated with idarubicin plus Ara-C, idarubicin plus Ara-C and an AKT inhibitor, or AKT inhibitor alone for 24 h, and ECAR and OCR were assessed using an *xf24* analyzer; cell viability was also measured using Annexin V/PI staining and CCK8 assays. Consistent with the results presented in Fig. 4, we found that treatment with AKT inhibitor decreased glycolysis, whereas treatment with idarubicin and AraC did not (Fig. 5A). In addition, co-treatment with AKT inhibitor and idarubicin and AraC further reduced glycolysis, glycolytic capacity, and glycolytic reserve. Interestingly, the three-drug combination (idarubicin, AraC and AKT inhibitor) reduced basal OCR, whereas treatment with individual drugs produced little change in OCR (Fig. 5B). To determine the cell apoptotic ratio, Annexin V/PI staining was used in conjunction with flow cytometry to quantify the apoptotic rate of KG1α cells following treatment with idarubicin, AraC, and AKT inhibitor. Although CCK8 assays revealed that cell viability was reduced by treatment with idarubicin and AraC (4.2%) or AKT inhibitor (14.4%), co-treatment with AKT inhibitor and idarubicin and AraC further reduced viability (26.6%) (Fig. 5C), without increasing Annexin V-stained and Annexin V/PI double-stained apoptotic cells (Fig. 5D). In conclusion, AKT inhibition enhances chemotherapeutic susceptibility in KG1α cells by suppressing the AKT signaling pathway and decreasing proliferative potential without inducing apoptosis.

Discussion

Resistance to intensive therapy is a major hurdle in the treatment of AML (36). Several new therapeutic approaches targeting mutant genes have been tested in an effort to overcome drug resistance (36,37). Despite such efforts, however, the molecular mechanisms responsible for refractory drug responses remain unidentified. Importantly, treatments based on the different properties of R/R AML cells must be developed as a common chemotherapy for AML. The results of the present study suggest that inhibiting the activity of AKT, which is a prominent metabolic modulator in R/R AML, is a promising new therapeutic strategy.

Inhibition of anaerobic glycolysis is important in relapse and chemoresistance. For example, chemoresistant colon cancer cells exhibit defective mitochondrial ATP production and enhanced aerobic glycolysis (38). It has also been demonstrated that glycolysis is increased and the efficiency of mitochondrial oxidative phosphorylation is reduced in drug-resistant HL60 ADR cells compared with the drug-sensitive HL60 cells (39). Consistent with those findings, the results of the present study demonstrated that glycolysis and mitochondrial oxidative phosphorylation activity were both increased in KG1α cells, which exhibited resistance to idarubicin and AraC, compared with control HL60 cells. Our data further suggested that this phenotype of KG1α cells may result from downregulation of PTEN, which is mutated in these cells.

Although PTEN mutations are rare in AML patients, they have been found to be correlated with refractory AML (20). Clinically, AML patients with a low level of PTEN and a high level of cyclin D1 expression have a high rate of relapse within 1 year (40). In addition, elderly PTEN-positive, CD44-negative AML patients survive significantly longer compared with PTEN-negative, CD44-positive patients (21). Although the association between PTEN mutation/expression and R/R AML has been investigated, the metabolic status of PTEN-mutated, refractory AML cells has not been fully elucidated. The present study demonstrated that PTEN affects metabolic flow and cell viability in refractory AML cell lines through inhibition of its downstream target, AKT. These results indicate that PTEN is a regulator of refractory AML cells through enhancement of glycolysis.

LSCs, which are capable of producing identical daughter cells as well as differentiated cells, are considered a major

cause of relapse and a serious obstacle to the successful treatment of AML (41). As cells of the KG1α leukemic cell line are considered to be LSC-like, they may also be used to evaluate whether the differential metabolic flow in LSCs underlies R/R AML. The metabolism of cancer cells is remodeled from oxidative phosphorylation to aerobic glycolysis even under aerobic conditions, a process termed the 'Warburg effect', which serves to support rapid growth and avoid hypoxia (42). The Warburg effect was initially hypothesized to result from diminished mitochondrial function (43); however, despite utilizing aerobic glycolysis, most cancer cells also consume oxygen (44,45). Activation of mitochondrial metabolism through mitochondrial biogenesis and quality control has been reported to maintain the supply of macromolecules to cancer cells (46). Cancer cells use glutamate as an important alternative carbon source to glucose. Previous experimental studies have demonstrated that cancer cells use glutamate for protein anabolism, and that Krebs cycle intermediates, through glutaminolysis, may act as building blocks for macromolecules necessary for growth and proliferation (47,48). Moreover, glutamine-driven oxidative phosphorylation supports ATP production in murine renal epithelial cells (49). In leukemia, LSC-positive cells are dependent on oxidative respiration rather than glycolysis for energy generation (11). AraC-resistant pre-existing and persisting cells display increased mitochondrial mass and retain active polarized mitochondria; this is consistent with a high oxidative phosphorylation status and not an LSC phenotype (13). In addition, upregulation of oxidative metabolism supports the survival of primitive CML cells, and combination treatment with imatinib and tigecycline, an antibiotic that inhibits mitochondrial protein translation, selectively eradicates CML LSCs *in vitro* as well as *in vivo* (50). KG1α cells with a highly oxidative respiratory capacity display characteristics of LSC-like cells and expression of the refractory AML phenotype. This observation highlights the need for further studies on how the regulation of mitochondrial function contributes to the survival of PTEN mutant, refractory leukemia cells.

In clinical trials, AKT inhibitors have proven to be highly toxic or cause only incomplete response in patients with advanced acute leukemia (51). The present study demonstrated that conventional chemotherapy together with AKT inhibitors, but not AKT inhibitors alone, reduce the viability of refractory AML cells by reducing mitochondrial OCR and glycolysis. Taken together, these findings suggest that metabolic deterioration mediated by AKT inhibition is involved in promoting susceptibility to the drug-refractory AML phenotype, providing a rationale for further study of targeted therapy (Fig. 6).

Acknowledgements

Not applicable.

Funding

The present study was supported by National Research Foundation of Korea (NRF) grants funded by the Ministry of Science and ICT (MSIT) (nos. 2016R1A6A3A11935284, 2016R1A2B4010398 and 2017R1A5A2015385 and 2019M3E5D1A02068575) and by the Ministry of Education (nos. 2014R1A6A1029617 and 2016R1D1A1B03932766).

Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

MJR, JYH and GRK made substantial contributions to the conception and design of the study. MJR was responsible for the acquisition of data and JH provided materials for the experiments. MJR and JYH helped with the analysis and interpretation of the data. MJR, XJ, WC, JYH and GRK wrote the manuscript. SJK, MJL, YLL, JHS, JC, YSJ and ICS contributed to the discussion and revised the article, and approved the final version of the manuscript. GRK and JYH were responsible for the integrity of the work as a whole. All the authors have read and approved the final version of the manuscript for publication.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Döhner H, Weisdorf DJ and Bloomfield CD: Acute Myeloid Leukemia. N Engl J Med 373: 1136-1152, 2015.
2. Gale RP: Advances in the treatment of acute myelogenous leukemia. N Engl J Med 300: 1189-1199, 1979.
3. Sakashita A, Hattori T, Miller CW, Suzushima H, Asou N, Takatsuki K and Koeffler HP: Mutations of the p53 gene in adult T-cell leukemia. Blood 79: 477-480, 1992.
4. Kimby E, Nygren P and Glimelius B; SBU-group: Swedish Council of Technology Assessment in Health Care: A systematic overview of chemotherapy effects in acute myeloid leukaemia. Acta Oncol 40: 231-252, 2001.
5. Mohammadi M, Cao Y, Glimelius I, Bottai M, Eloranta S and Smedby KE: The impact of comorbid disease history on all-cause and cancer-specific mortality in myeloid leukemia and myeloma-a Swedish population-based study. BMC Cancer 15: 850, 2015.
6. De Kouchkovsky I and Abdul-Hay M: Acute myeloid leukemia: A comprehensive review and 2016 update'. Blood Cancer J 6: e441, 2016.
7. Stone RM, Moser B, Sanford B, Schulman P, Kolitz JE, Allen S, Stock W, Galinsky I, Vij R, Marcucci G, Hurd D, *et al*: High dose cytarabine plus gemtuzumab ozogamicin for patients with relapsed or refractory acute myeloid leukemia: Cancer and leukemia Group B study 19902. Leuk Res 35: 329-333, 2011.
8. Aziz H, Ping CY, Alias H, Ab Mutalib NS and Jamal R: Gene mutations as emerging biomarkers and therapeutic targets for relapsed acute myeloid leukemia. Frontiers in Pharmacology 8: 897, 2017.
9. Levis M: Midostaurin approved for FLT3-mutated AML. Blood 129: 3403-3406, 2017.

10. Stein EM, DiNardo CD, Pollyea DA, Fathi AT, Roboz GJ, Altman JK, Stone RM, DeAngelo DJ, Levine RL, Flinn IW, *et al*: Enasidenib in mutant *IDH2* relapsed or refractory acute myeloid leukemia. Blood 130: 722-731, 2017.
11. Lagadinou ED, Sach A, Callahan K, Rossi RM, Neering SJ, Minhajuddin M, Ashton JM, Pei S, Grose V, O'Dwyer KM, *et al*: BCL-2 inhibition targets oxidative phosphorylation and selectively eradicates quiescent human leukemia stem cells. Cell Stem Cell 12: 329-341, 2013.
12. Škrtić M, Sriskanthadevan S, Jhas B, Gebbia M, Wang X, Wang Z, Hurren R, Jitkova Y, Gronda M, Maclean N, *et al*: Inhibition of mitochondrial translation as a therapeutic strategy for human acute myeloid leukemia. Cancer Cell 20: 674-688, 2011.
13. Kuntz EM, Baquero P, Michie AM, Dunn K, Tardito S, Holyoake TL, Helgason GV and Gottlieb E: Targeting mitochondrial oxidative phosphorylation eradicates therapy-resistant chronic myeloid leukemia stem cells. Nat Med 23: 1234-1240, 2017.
14. Manning BD and Toker A: AKT/PKB Signaling: Navigating the network. Cell 169: 381-405, 2017.
15. Hawkins PT and Stephens LR: PI3K signalling in inflammation. Biochim Biophys Acta 1851: 882-897, 2015.
16. Zhao HF, Wang J and Tony To SS: The phosphatidylinositol 3-kinase/Akt and c-Jun N-terminal kinase signaling in cancer: Alliance or contradiction? (Review). Int J Oncol 47: 429-436, 2015.
17. Kang YH, Lee HS and Kim WH: Promoter methylation and silencing of PTEN in gastric carcinoma. Lab Invest 82: 285-291, 2002.
18. García JM, Silva J, Peña C, Garcia V, Rodríguez R, Cruz MA, Cantos B, Provencio M, España P and Bonilla F: Promoter methylation of the PTEN gene is a common molecular change in breast cancer. Genes Chromosomes Cancer 41: 117-124, 2004.
19. Alvarez-Nunez F, Bussaglia E, Mauricio D, Ybarra J, Vilar M, Lerma E, de Leiva A and Matias-Guiu X; Thyroid Neoplasia Study Group: PTEN promoter methylation in sporadic thyroid carcinomas. Thyroid 16: 17-23, 2006.
20. Liu TC, Lin PM, Chang JG, Lee JP, Chen TP and Lin SF: Mutation analysis of PTEN/MMAC1 in acute myeloid leukemia. Am J Hematol 63: 170-175, 2000.
21. Huang X, Li D, Li T, Zhao BO and Chen X: Prognostic value of the expression of phosphatase and tensin homolog and CD44 in elderly patients with refractory acute myeloid leukemia. Oncol Lett 10: 103-110, 2015.
22. Yilmaz OH, Valdez R, Theisen BK, Guo W, Ferguson DO, Wu H and Morrison SJ: Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. Nature 441: 475-482, 2006.
23. Zhang J, Grindley JC, Yin T, Jayasinghe S, He XC, Ross JT, Haug JS, Rupp D, Porter-Westpfahl KS, Wiedemann LM, *et al*: PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. Nature 441: 518-522, 2006.
24. Fragoso R and Barata JT: PTEN and leukemia stem cells. Adv Biol Regul 56: 22-29, 2014.
25. Wang L, Xiong H, Wu F, Zhang Y, Wang J, Zhao L, Guo X, Chang LJ, Zhang Y, You MJ, *et al*: Hexokinase 2-mediated Warburg effect is required for PTEN- and p53-deficiency-driven prostate cancer growth. Cell Rep 8: 1461-1474, 2014.
26. Li Y, He L, Zeng N, Sahu D, Cadenas E, Shearn C, Li W and Stiles BL: Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) signaling regulates mitochondrial biogenesis and respiration via estrogen-related receptor α (ERRα). J Biol Chem 288: 25007-25024, 2013.
27. Pietkiewicz S, Schmidt JH and Lavrik IN: Quantification of apoptosis and necroptosis at the single cell level by a combination of Imaging Flow Cytometry with classical Annexin V/propidium iodide staining. J Immunol Methods 423: 99-103, 2015.
28. She M, Niu X, Chen X, Li J, Zhou M, He Y, Le Y and Guo K: Resistance of leukemic stem-like cells in AML cell line KG1a to natural killer cell-mediated cytotoxicity. Cancer Lett 318: 173-179, 2012.
29. Wiernik PH, Banks PL, Case DC Jr, Arlin ZA, Periman PO, Todd MB, Ritch PS, Enck RE and Weitberg AB: Cytarabine plus idarubicin or daunorubicin as induction and consolidation therapy for previously untreated adult patients with acute myeloid-leukemia. Blood 79: 313-319, 1992.
30. Gallay N, Dos Santos C, Cuzin L, Bousquet M, Simmonet Gouy V, Chaussade C, Attal M, Payrastre B, Demur C and Récher C: The level of AKT phosphorylation on threonine 308 but not on serine 473 is associated with high-risk cytogenetics and predicts poor overall survival in acute myeloid leukaemia. Leukemia 23: 1029-1038, 2009.
31. Elstrom RL, Bauer DE, Buzzai M, Karnauskas R, Harris MH, Plas DR, Zhuang H, Cinalli RM, Alavi A, Rudin CM and Thompson CB: Akt stimulates aerobic glycolysis in cancer cells. Cancer Res 64: 3892-3899, 2004.
32. Barthel A, Okino ST, Liao J, Nakatani K, Li J, Whitlock JP Jr and Roth RA: Regulation of GLUT1 gene transcription by the serine/threonine kinase Akt1. J Biol Chem 274: 20281-20286, 1999.
33. Xu DD, Wang Y, Zhou PJ, Qin SR, Zhang R, Zhang Y, Xue X, Wang J, Wang X, Chen HC, *et al*: The IGF2/IGF1R/nanog signaling pathway regulates the proliferation of acute myeloid leukemia stem cells. Front Pharmacol 9: 687, 2018.
34. Matkovic K, Brugnoli F, Bertagnolo V, Banfic H and Visnjic D: The role of the nuclear Akt activation and Akt inhibitors in all-trans-retinoic acid-differentiated HL-60 cells. Leukemia 20: 941-951, 2006.
35. Thol F, Schlenk RF, Heuser M and Ganser A: How I treat refractory and early relapsed acute myeloid leukemia. Blood 126: 319-327, 2015.
36. Bose P, Vachhani P and Cortes JE: Treatment of relapsed/refractory acute myeloid leukemia. Curr Treat Options Oncol 18: 17, 2017.
37. Zhou Y, Tozzi F, Chen J, Fan F, Xia L, Wang J, Gao G, Zhang A, Xia X, Brasher H, *et al*: Intracellular ATP levels are a pivotal determinant of chemoresistance in colon cancer cells. Cancer Res 72: 304-314, 2012.
38. Song K, Li M, Xu X, Xuan LI, Huang G and Liu Q: Resistance to chemotherapy is associated with altered glucose metabolism in acute myeloid leukemia. Oncol Lett 12: 334-342, 2016.
39. Tanioka M, Sakai K, Sudo T, Sakuma T, Kajimoto K, Hirokaga K, Takao S, Negoro S, Minami H, Nakagawa K and Nishio K: Transcriptional CCND1 expression as a predictor of poor response to neoadjuvant chemotherapy with trastuzumab in HER2-positive/ER-positive breast cancer. Breast Cancer Res Treat 147: 513-525, 2014.
40. Jordan CT: The leukemic stem cell. Best Pract Res Clin Haematol 20: 13-18, 2007.
41. Vander Heiden MG, Cantley LC and Thompson CB: Understanding the Warburg effect: The metabolic requirements of cell proliferation. Science 324: 1029-1033, 2009.
42. Warburg O: On the origin of cancer cells. Science 123: 309-314, 1956.
43. Weinhouse S: On respiratory impairment in cancer cells. Science 124: 267-269, 1956.
44. Zu XL and Guppy M: Cancer metabolism: Facts, fantasy, and fiction. Biochem Biophys Res Commun 313: 459-465, 2004.
45. Li X, Jiang Y, Meisenhelder J, Yang W, Hawke DH, Zheng Y, Xia Y, Aldape K, He J, Hunter T, *et al*: Mitochondria-translocated PGK1 functions as a protein kinase to coordinate Glycolysis and the TCA Cycle in tumorigenesis. Mol Cell 61: 705-719, 2016.
46. Mates JM, Segura JA, Campos-Sandoval JA, Lobo C, Alonso L, Alonso FJ and Márquez J: Glutamine homeostasis and mitochondrial dynamics. Int J Biochem Cell Biol 41: 2051-2061, 2009.
47. Meng M, Chen S, Lao T, Liang D and Sang N: Nitrogen anabolism underlies the importance of glutaminolysis in proliferating cells. Cell Cycle 9: 3921-3932, 2010.
48. Fan J, Kamphorst JJ, Mathew R, Chung MK, White E, Shlomi T and Rabinowitz JD: Glutamine-driven oxidative phosphorylation is a major ATP source in transformed mammalian cells in both normoxia and hypoxia. Mol Syst Biol 9: 712, 2013.
49. Farge T, Saland E, de Toni F, Aroua N, Hosseini M, Perry R, Bosc C, Sugita M, Stuani L, Fraisse M, Scotland S, *et al*: Chemotherapy-resistant human acute myeloid leukemia cells are not enriched for leukemic stem cells but require oxidative metabolism. Cancer Discov 7: 716-735, 2017.
50. Gojo I, Perl A, Luger S, Baer MR, Norsworthy KJ, Bauer KS, Tidwell M, Fleckinger S, Carroll M and Sausville EA: Phase I study of UCN-01 and perifosine in patients with relapsed and refractory acute leukemias and high-risk myelodysplastic syndrome. Invest New Drugs 31: 1217-1227, 2013.
51. Sampath D, Malik A, Plunkett W, Nowak B, Williams B, Burton M, Verstovsek S, Faderl S, Garcia-Manero G, List AF, *et al*: Phase I clinical, pharmacokinetic, and pharmacodynamic study of the Akt-inhibitor triciribine phosphate monohydrate in patients with advanced hematologic malignancies. Leuk Res 37: 1461-1467, 2013.