PI3K/AKT/mTOR pathway regulates aerobic glycolysis

Signaling through the Phosphatidylinositol 3-Kinase (PI3K)/ Mammalian Target of Rapamycin (mTOR) Axis is Responsible for Aerobic Glycolysis mediated by Glucose Transporter in Epidermal Growth Factor Receptor (EGFR)-mutated Lung Adenocarcinoma

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Keywords: EGFR, glycolysis, lung adenocarcinoma, glucose transporter, metabolic checkpoint

Background: EGFR signaling maintains aerobic glycolysis but, the molecular mechanism is still undefined.

Results: Drug inhibition studies reveal that downstream signaling via the PI3K pathway is critical for glucose transport and metabolism.

Conclusion: The PI3K signaling regulates key metabolic activities in EGFR-mutant lung adenocarcinoma.

Significance: These data may guide the development of chemotherapeutic options, including targeting of the PI3K pathway and glucose transporter machinery.

Oncogenic epidermal growth factor receptor (EGFR) signaling plays an important role in regulating global metabolic pathways including aerobic glycolysis, the pentose phosphate pathway (PPP) and pyrimidine biosynthesis. However, the molecular mechanism by which EGFR signaling regulates cancer cell metabolism is still unclear. To elucidate how EGFR signaling is linked to metabolic activity, we investigated the involvement of the RAS/MEK/ERK and PI3K/akt/mTOR pathways on metabolic alteration in lung adenocarcinoma (LAD) cell lines with activating EGFR mutations. Although MEK inhibition did not alter lactate production and the extracellular acidification rate (ECAR), PI3K/mTOR inhibitors significantly suppressed glycolysis in EGFR-mutant LAD cells. Moreover, comprehensive metabolomics analysis revealed that the levels of glucose 6-phosphate (G6P) and 6-phosphogluconate (6PG) as early metabolites in glycolysis and PPP were decreased after inhibition of the PI3K/akt/mTOR pathway, suggesting a linkage between PI3K signaling and the proper function of glucose transporters or hexokinases in glycolysis. Indeed, PI3K/mTOR inhibition effectively suppressed membrane localization of facilitative glucose transporter 1 (GLUT1), which instead accumulated in the cytoplasm. Finally, aerobic glycolysis and cell proliferation were down-regulated when GLUT1 gene expression was suppressed by RNA interference (RNAi). Taken together, these results suggest that PI3K/akt/mTOR signaling is indispensable for the regulation of aerobic glycolysis in EGFR-mutated LAD cells.

Cancer genomics using next generation sequencing (NGS) technology have successfully characterized a number of therapeutic targets of lung cancer (1-6). EGF mutations were identified in more than 50% of lung adenocarcinomas (LAD) from East Asian non-smokers, and these tumors have been termed...
The PI3K/AKT/mTOR pathway is responsible for LAD. These findings indicate that the production and cell proliferation in blocking with siRNA resulted in decreased lactate glycolysis in that, of these two pathways, the PI3K/AKT/mTOR pathways (8,9). Here we show EGFR signaling via the RAS/MEK/ERK and dysregulated by gene mutations that lead to aberrant this scenario, the tyrosine kinase activity of EGFR is predominantly expressed in the small intestine and tumor progression (19,20,23). The SGLT family is shown to play a role in cancer cell metabolic reprogramming (19,20). At present, two classes of glucose transporters, GLUTs and sodium-glucose symporters (SGLTs), exist in cancer cells (21,22). The GLUT (SLC2A) family is a wide group of membrane proteins including 14 hexose transporters that facilitate the transport of glucose over the plasma membrane (21,22). In some cancers, GLUT1 overexpression was found to be associated with tumor progression (19,20,23). The SGLT family is predominantly expressed in the small intestine and the kidney. One report has suggested that EGFR physically associates with and stabilizes SGLT1 to promote glucose uptake into cancer cells (24). Previously, we have shown that mutant EGFR signaling maintains up-regulated aerobic glycolysis, PPP and de novo pyrimidine biosynthesis (11). In this scenario, the tyrosine kinase activity of EGFR is dysregulated by gene mutations that lead to aberrant EGFR signaling via the RAS/MEK/ERK and PI3K/AKT/mTOR pathways (8,9). Here we show that, of these two pathways, the PI3K/AKT/mTOR signaling axis plays a more critical role in regulating glycolysis in EGFR-mutated LAD, and appears to facilitate the proper localization of the glucose transporter GLUT1. Suppression of GLUT1 after blocking with siRNA resulted in decreased lactate production and cell proliferation in EGFR mutated LAD. These findings indicate that the PI3K/AKT/mTOR pathway is responsible for aerobic glycolysis by regulating GLUT1 localization in EGFR-mutated LAD.

**EXPERIMENTAL PROCEDURES**

**Purchased materials**-Cell lines were purchased from Immuno-Biological Laboratories (Fujiko, Japan) and American Type Culture Collection. RPMI-1640 (R8758 and R1383), phosphate-buffered saline (PBS), 2-deoxy-D-glucose (2DG) were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was purchased from BioWest (Nuaille, France). Erlotinib (ERLO), AZD6244, BEZ235 (BEZ), AZD9291 and PKI-587 were purchased from Selleck (Houston, TX). EnVision TM+, Peroxidase–conjugated anti-Rabbit and antibody diuluent were purchased from Dako (Glostrup, Denmark). Dimethyl sulfoxide (DMSO), glucose and 3, 3-diaminobenzidine (DAB) were purchased from Wako Pure Chemicals Industries (Osaka, Japan). Cell Counting Kit-8 was purchased from Dojindo Laboratories (Kumamoto, Japan). Lactate assay kit II was purchased from BioVision (Milpitas, CA). FluxPak XF24 assay pack and XF glycolysis stress test kit were purchased from Seahorse Bioscience (North Billerica, MA). Countess Automated Cell Counter including Trypan Blue and chamber slides was purchased from Invitrogen (Carlsbad, CA). Mini-PROTEAN TGX Precast Gel, Trans-Blot Turbo Transfer System and Trans-Blot Turbo Transfer Pack were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). Primary antibodies specific for EGFR, phospho-EGFR (pEGFR), AKT, phospho-AKT (pAKT), ERK1/2, phospho-ERK1/2 (pERK) and beta-actin were purchased from Cell Signaling Technologies (Danvers, MA). Anti-GLUT1 and anti-Na/K were purchased from Abcam (Cambridge, UK). GLUT1-targeting siRNA and BCA protein assay were purchased from Thermo Fisher Scientific (Waltham, MA). The peroxidase-linked secondary antibodies for Western blot, HRP-linked sheep anti-mouse IgG and donkey anti-rabbit IgG, were purchased from GE Healthcare (Wauwatosa, WI). Alexa488-conjugated anti-rabbit IgG and Alexa594-conjugated anti-mouse IgG were purchased from Invitrogen (Carlsbad, CA). Oligomycin was purchased from Merck Millipore (Darmstadt, Germany). VECTASHIELD Mounting Medium was purchased from VECTOR LABORATORIES (Burlingame, CA). FITC-conjugated anti-rabbit IgG antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

**Cell survival assay and proliferation assay**-EGFR mutant LAD cells were seeded in RPMI 1640 containing various concentrations of inhibitors in 96-well cell culture plates. After 72 hour incubation...
at 37°C 5% CO₂, cell viability was analyzed by the WST-8 assay using the Cell Counting Kit-8. The optical density of the cell culture medium in each well was read at 450 nm on a microplate reader (Molecular Devices, Sunnyvale, CA). Viable cells were enumerated by trypan blue exclusion using a Countess Automated Cell Counter (Life Technologies).

**Western Blotting**-Cells were lysed in RIPA buffer on ice for 10 min and centrifuged at 15,000 × g for 10 min. The protein content of supernatants was quantified by BCA assay (Pierce). Proteins were separated on a commercially available 4%-20% gradient SDS-polyacrylamide gel (Mini-PROTEAN TGX; Bio-Rad Laboratories, Inc.) and transferred to a polyvinylidene difluoride membrane (Trans-Blot Turbo Transfer Pack; Bio-Rad Laboratories, Inc.). They were then incubated overnight with primary antibodies (1:1000). The primary antibodies used in this study are described above. ECL anti-rabbit IgG HRP-linked whole antibody (GE Healthcare, 1:10,000) was used as secondary antibody, and signals were detected using ECL Western Blotting Detection reagent (GE Healthcare) and X-ray films (GE Healthcare). β-actin was used as loading control.

**Lactate assay**-Lactate in culture medium was quantified with a lactate assay kit II according to the manufacturer's instructions (Biovision, Mountain View, CA). The lactate concentration was quantified by BCA assay (Pierce). Proteins were separated on a commercially available 4%-20% gradient SDS-polyacrylamide gel (Mini-PROTEAN TGX; Bio-Rad Laboratories, Inc.) and transferred to a polyvinylidene difluoride membrane (Trans-Blot Turbo Transfer Pack; Bio-Rad Laboratories, Inc.). They were then incubated overnight with primary antibodies (1:1000). The primary antibodies used in this study are described above. ECL anti-rabbit IgG HRP-linked whole antibody (GE Healthcare, 1:10,000) was used as secondary antibody, and signals were detected using ECL Western Blotting Detection reagent (GE Healthcare) and X-ray films (GE Healthcare). β-actin was used as loading control.

**Measurement of ECAR and oxygen consumption rate (OCR)**-ECAR and OCR were measured with a XF glycolysis stress test kit according to the manufacturer's instructions (Seahorse Bioscience). In brief, 4.5 × 10⁴ cells were plated onto XF24 plates in RPMI-1640 (10% FBS, 2 mM glutamine) and incubated at 37°C, 5% CO₂ overnight. Medium including DMSO or inhibitors were placed into each well and cells were incubated for 6 hours. Cells were washed with assay medium (minus glucose and unbuffered RPMI-1640 (SIGMA R1383)), replaced with assay medium and then placed at 37°C in a CO₂-free incubator for 30 min. ECAR and OCR were measured using a Seahorse Bioscience XF24 Extracellular Flux Analyzer over time. Each cycle consisted of 3 min mixing, 3 min waiting, and 3 min measuring. Glucose, oligomycin and 2DG were diluted into XF24 media and loaded into the accompanying cartridge to achieve final concentrations of 10 mM, 5 μM, and 100 mM respectively.

**Metabolite Measurements**-H1975 cells were grown in RPMI-1640 containing 11.1 mM [U-13C] glucose (2-13C-Glc₃) in the presence of DMSO, AZD9291 or PKI-587. Metabolic extracts were prepared after 6 hour incubation and analyzed using a capillary electrophoresis (CE)-connected ESI-TOFMS and CE-MS/MS system (Human Metabolome Technologies; HMT, Inc., Tsuruoka, Japan, F-SCOPE) (25,26). For quantitative static metabolomic analysis, samples were prepared from 2 – 5 × 10⁶ cells with methanol containing internal standard solution (HMT) and analyzed using a CE-connected ESI-TOFMS and CE-MS/MS system (HMT, C-SCOPE). Detailed procedures were published in our previous paper (11).

**Immunohistochemistry (IHC)**-All IHC analyses were performed on paraffin-embedded tissues obtained from the primary tumor in the surgical specimen. This study was approved by the Institutional Review Board (IRB) of the National Cancer Center, Japan (IRB number: 2014-325). All EGFR mutation status information used in this study was obtained from a database at the Division of Thoracic Oncology, National Cancer Center Hospital East, Kashiwa, Japan. We prepared and used 4-μm-thick paraffin sections cut from a paraffin block containing histological findings that were representative of the tumor. Antigen retrieval was performed in citrate buffer solution (pH 6.0). Endogenous peroxidase was blocked with 0.3% H₂O₂ in methanol for 15 min and all slides were heated to 95°C by exposure to microwave irradiation for 20 min. Slides were washed in PBS and after a 1 h incubation at RT with the primary antibodies, the slides were incubated for 30 min with a labeled polymer EnVision TM+, peroxidase–conjugated anti-rabbit antibody (Dako, Tokyo, Japan). The chromogen used was 2% 3,3-diaminobenzidine (DAB) in 50 mM Tris-buffer (pH 7.6) containing 0.3% hydrogen. **Immunofluorescence**-Cells were seeded in ibidi 35 mm µ-Dish (Maitai, Germany) and cultured overnight. They were then incubated with inhibitors for 6 hours. Cells were washed three times and fixed with 4% paraformaldehyde for 15 min. After washing, the fixed cells were treated with blocking buffer (1% bovine serum albumin (BSA), 3% TritonX-100, PBS) and incubated with primary antibody at 4°C overnight. After washing, cells were incubated in secondary antibody (Alexa488-conjugated anti-rabbit IgG and Alexa594-conjugated anti-mouse IgG) for 1 h, stained with DAPI, mounted with VECTASTIELD Mounting Medium, and analyzed using the confocal microscopy Laser Scanning Microscope 710 (LSM710, Carl Zeiss, Oberkochen, Germany).
Flow cytometric analysis-To quantitatively detect expression of membrane-bound GLUT1, cells were fixed with 80% ethanol and incubated with anti-GLUT1 antibody (Abcam) and then stained with the appropriate FITC-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch). Quantification of FITC-fluorescent intensity was performed using a FACSCanto II (BD Biosciences). siRNA transfection-LAD cells (1.0 × 10^6) were transfected with 5 nM siGLUT1#1 (s12925, Thermo Fisher Scientific), siGLUT1#2 (s12926, Thermo Fisher Scientific) or siNC (4390843, Thermo Fisher Scientific) using Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific).

Statistical Analyses-Unless otherwise indicated, results were reported as the mean ± S.D. Statistical analyses were done by two-tailed Student t test.

RESULTS

EGFR-mutant LAD cells were more sensitive to dual PI3K/mTOR inhibitors than MEK inhibitor-We studied three lung adenocarcinoma cell lines that possessed the EGFR mutation in either exon 19 or exon 21. Cell lines HCC827 and PC-9 both carried the delE746-A750 mutation, while NCI-H1975 carried EGFR L858R plus T790M, a mutation which confers resistance to erlotinib. First, we confirmed that the HCC827 and PC-9 cell lines were highly sensitive to the EGFR-TKI, erlotinib (Fig. 1, A and B) with IC50 values in the nanomolar range as compared to the erlotinib-resistant H1975 (Fig. 1, C). We also treated H1975 cells with AZD9291, a selective third generation irreversible EGFR inhibitor of T790M resistance mutants (27). Here we confirmed that AZD9291 reduced viability in H1975 cells to a greater extent as compared to erlotinib after 3 days of treatment (Fig. 1, D).

Several signaling pathways related to cellular proliferation and survival are activated downstream of EGFR, including the RAS/MEK/ERK and PI3K/ AKT/mTOR pathways. AZD6244 (Selumetinib) is a non-ATP competitive inhibitor and highly specific for MEK, a key enzyme in the RAS/RAF/MEK/ERK pathway, while NVP-BEZ235 (Dactolisib) and PKI-587 (Gedatolisib) are dual pan PI3K and mTOR inhibitors. As compared to MEK inhibitor AZD6244 (IC50=>10 μM), treatment with PI3K/mTOR inhibitor BEZ235 inhibited growth of all three cell lines more effectively with similar dose-dependent decreases in viability (mean IC50=11, 5.0, and 42 nM for HCC827, PC-9 and H1975 cell lines) (Fig. 1 A-C). In addition, PKI-587 also effectively kills H1975 cells harboring the T790M resistance mutation (Fig. 1, D).

Altered phosphorylation of EGFR signaling proteins in EGFR-mutant LAD cells after treatment with inhibitors- To verify that the compounds used inhibited the corresponding signaling pathways, phosphorylation of EGFR signaling molecules was investigated by Western blot (WB) analysis in HCC827, PC-9, and H1975 cells (Fig. 2). We examined the effect of the inhibitors on molecular markers of the EGFR signaling cascade (EGFR, AKT and ERK) in LAD cells incubated in the presence of erlotinib, AZD6244, BEZ235, AZD9291 and PKI-587, respectively. WB analysis showed that phosphorylation of EGFR (pEGFR), AKT (pAKT), and ERK (pERK) was inhibited at 6 hours after erlotinib treatment in HCC827 and PC9 cells (Fig. 2). Alterations in any of the major EGFR signaling cascades were not observed in EGFR TKI-resistant H1975 cells treated with erlotinib, while treatment with AZD9291 down-regulated phosphorylation of EGFR and downstream signaling (Fig. 2). AZD6244 treatment inhibited the RAS/MEK/ERK pathway and slightly increased AKT phosphorylation in all three cell lines (Fig. 2). Signaling through the PI3K/AKT/mTOR pathway was down-regulated in all three cell lines in the presence of BEZ235 and PKI-587 (Fig. 2) as shown by decreased pAKT, consistent with previous reports (28,29).

Glycolytic activities decreased after inhibition of PI3K/AKT/mTOR, but not RAS/MEK/MAPK, pathway in EGFR-mutant LAD cells-Treatment with EGFR TKIs decreases lactate production, glucose consumption, and the glucose-induced extracellular acidification rate (ECAR) in LAD cells, indicating that EGFR signaling maintains aerobic glycolysis (11). By using specific inhibitors to block either the RAS/MEK/ERK or PI3K/AKT/mTOR pathway, we observed that a 72-hr incubation with dual PI3K/mTOR inhibitors led to a dramatic reduction in cell viability in BEZ235-sensitive EGFR-mutant cell lines, suggesting the PI3K/AKT/mTOR pathway is critical for aerobic glycolysis downstream of EGF (Fig. 1). To examine this further, we set up experimental conditions where inhibitor treatment was given at a relatively higher concentration (1 μM) and shorter time frame (6 hours), a method we had previously utilized for EGFR-TKI treatment (11). Under this experimental condition, all cells grew equally and thereby standardized the number of viable cells analyzed (Fig. 3, A). Our results showed that lactate production was decreased in HCC827 and PC-9 cells after treatment with erlotinib, but not changed in H1975 cells (Fig. 3, B). Moreover, we discovered that exposure of the cells to PI3K/mTOR inhibitors for up to 6 hours significantly lowered the rate of lactate accumulation in the medium of LAD cell lines, while cells treated with MEK inhibitor did not show this effect (Fig. 3, B). To better define lactate production derived from glucose, we measured...
ECAR, an indicator of lactate production, and the oxygen consumption rate (OCR), an indicator of oxidative phosphorylation (OXPHOS), using a flux analyzer. ECAR was statistically higher in DMSO controls and after AZD6244 MEK inhibitor treatment, as compared to PI3K/mTOR inhibitor-treated HCC827, PC-9 and H1975 cells (Figure 3b). In contrast to ECAR, OCR was not significantly changed in HCC827, PC-9 and H1975 cells under this experimental condition (data not shown). Treatment with the third generation EGFR-TKI AZD9291 caused reduction of both lactate production and ECAR in H1975 cells (Fig. 3, B and C). These data suggest that PI3K/AKT/mTOR signaling plays a crucial role in maintenance of aerobic glycolysis in EGFR-mutant LAD cells.

**PI3K/AKT/mTOR signaling up-regulates glycolysis and the pentose phosphate pathway—**Our flux analysis indicated that glucose-derived lactate production could be reduced by inhibition of the PI3K/AKT/mTOR but not the RAS/MEK/MAPK pathway. We next used a metabolomics approach to quantify intracellular metabolites in LAD cells in the presence or absence of inhibitors. First, we tested the metabolic consequences of EGFR or PI3K/mTOR inhibition in H1975 cells given uniformly labeled [U-13C]-Glucose under our experimental conditions (Fig. 4, A). Although the levels of metabolites at steady state are the result of the balance between production and consumption, the use of the 13C-label allows us to determine not only steady levels of metabolites but also the isotopomer and isotopologue distributions of metabolites derived from 13C-labeled glucose for reconstruction of metabolic pathways (30,31). We measured the metabolites derived from 13C-glucose in glycolysis and PPP such as glucose 6-phosphate (G6P), 6-phosphogluconate (6PG) and lactate (Lac) in the presence of DMSO, AZD9291, and PKI-587 (Fig. 4, A). Repression of EGFR signaling in H1975 cells with AZD9291 resulted in a reduction of total and 13C-glucose-derived G6P, 6PG and fructose 1,6-bisphosphate (F1,6P), which are early metabolites in glycolysis and PPP. The impact of PKI-587 on metabolites in H1975 cells was similar to that for TKIs, except for several metabolites such as amino acids (Fig. 4, A). We also confirmed that the final product of glycolysis, total and 13C-lactate (m+3), were decreased after treatment with AZD9291 and PKI-587 (Fig. 4, A).

To quantify the reduction of metabolite amount at steady state, we extracted intracellular metabolites with methanol and analyzed using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS). Metabolome analysis revealed that the upstream metabolites in glycolysis and the PPP were reduced after PKI-587 treatment for 6 hours in both PC-9 and H1975 cells (Fig. 4, B and Supplementary Table 1). The carbamoyl-phosphate synthetase 2, aspartate transcarbamoylase, and dihydroorotase (CAD) protein is required for the first step in the de novo synthesis of pyrimidines, and this protein is activated by mTOR via ribosomal protein S6 kinase 1 (S6K) (32). As expected, carbamoyl aspartic acid (Carbamoyl-Asp) levels were decreased after PI3K/mTOR inhibitor treatment as determined by metabolome analysis (Fig. 4, B). In contrast, several amino acids were increased after inhibition of PI3K/AKT/mTOR signaling (Supplementary Table 1). Moreover, the reduction of intermediate metabolites in glycolysis and PPP, decreased Carbamoyl-Asp amount, and accumulating amino acids were reproduced in both H1975 cells treated with AZD9291 and HCC827 cells treated with PKI-587 (Supplementary Table 1). Taken together, metabolomics analysis indicates that the PI3K/AKT/mTOR signaling pathway is indispensable for aerobic glycolysis and de novo pyrimidine biosynthesis in EGFR-mutated LAD cells.

**PI3K/AKT/mTOR signaling maintains membrane localization of GLUT1**—Comprehensive metabolomics analysis suggested that glucose transporter and hexokinase activities could be regulated by PI3K/AKT/mTOR signaling. Our previous genomics analysis of mRNA expression in LAD cell lines indicated predominant up-regulation of the glucose transporter family member GLUT1 (SLC2A1) (33). To assess whether GLUT1 was expressed on the plasma membrane in EGFR-mutant LAD tissues, we performed immunohistochemical (IHC) analysis of 33 EGFR-mutant LAD cases. Representative IHC evaluations of cytosolic or membrane GLUT1-positivity with anti-GLUT1 antibody are shown (Fig. 5, A). We found that GLUT1 was predominantly localized in the cytosol (46 %) with some at the cell membrane (18%) (Fig. 5, B). Interestingly, 36% of cases were GLUT1-negative despite their EGFR-mutant status, suggesting that glucose transporters other than GLUT1 might be operating in such tumor tissues (Fig. 5, B). We next examined the effects of PI3K/mTOR inhibition on membrane-bound GLUT1 in HCC827 cells by immunofluorescence (Fig. 5, C). We observed a significant fraction of GLUT1 localized at the plasma membrane in HCC827 cells under normal culture conditions (Fig. 5, C). Upon 6 hour exposure to EGFR and PI3K/mTOR inhibitors, however, GLUT1 appeared as punctate structures distributed throughout the cell as well as in structures...
concentrated in the perinuclear region (Fig. 5, C). On the other hand, localization of GLUT1 was unchanged after 6 h of MEK inhibitor AZD6244-treatment (data not shown). Western blot analysis showed that total GLUT1 was not decreased in HCC827, PC-9, and H1975 cells after 6 h treatment with any inhibitors (Fig. 6, A). To determine the effects of erlotinib and BEZ235 on membrane expression of glucose transporters in HCC827, PC-9 and H1975 cells, we quantitatively measured cell surface GLUT1 levels by flow cytometry (Fig. 6, B-G). Representative flow cytometry plots of GLUT1 expression in HCC827 (Fig. 6, B), PC-9 (Fig. 6, C) and H1975 (Fig. 6, D) cells treated with DMSO or BEZ235 were shown. We observed reduction of membrane-bound GLUT1 in the erlotinib-sensitive cell lines HCC827 (Fig. 6, E) and PC-9 (Fig. 6, F) after 6-hr erlotinib treatment, while the expression of GLUT1 was unchanged in erlotinib-resistant H1975 cells (Fig. 6, G). On the other hand, treatment with BEZ235 down-regulated membrane GLUT1 levels in all three cell lines (Fig. 6, B-G). These results suggest that the PI3K/AKT/mTOR signaling pathway is required to maintain GLUT1 at the plasma membrane in EGFR-mutant LAD cells.

**Loss of GLUT1 decreases lactate production and cell growth**-To characterize further the function of GLUT1 in LAD cells, we employed a genetic approach to repress GLUT1 expression by RNA interference (RNAi). Western blot analyses revealed significant decreases in GLUT1 protein expression upon introduction of two targeting RNAi constructs, siGLUT1#1 and siGLUT1#2, as compared to a non-targeting control (siNC), in PC-9 and H1975 cells under normal culture conditions (Fig. 7, A). To measure glycolytic activity in siGLUT1-transfected LAD cells, we quantitated lactate in culture media. Loss of GLUT1 significantly lowered the rate of lactate accumulation in the medium of LAD cell lines (Fig. 7, B). Moreover, the number of PC-9 and H1975 cells significantly decreased 24 and 48 h after introduction of siGLUT1 (Fig. 7, C and D). Altogether, these results indicate that GLUT1 is important for glucose metabolism and survival/proliferation of LAD cells, and that the PI3K/AKT/mTOR signaling pathway appears to play a critical role in this process, likely by supporting the proper membrane localization of GLUT1 for optimal function in glycolysis.

**DISCUSSION**

In our previous paper, we showed that treatment with TKIs to EGFR, namely gefitinib and erlotinib, repressed aerobic glycolysis in EGFR-mutant LAD cells (11). Here we have expanded upon those results by demonstrating that the PI3K/AKT/mTOR signaling pathway downstream of EGFR maintains aerobic glycolysis through GLUT1 function in EGFR-mutant LAD cells. Regulation of GLUT1 localization by PI3K/AKT/mTOR signaling is important for supporting glycolysis and the pentose phosphate pathway, since loss of GLUT1 via suppression by siRNA to GLUT1 significantly lowered the rate of lactate accumulation and cellular growth of EGFR-mutant LAD cell lines. We conclude that signaling through the PI3K/AKT/mTOR pathway, but not RAS/MEK/ERK pathway, is responsible for aerobic glycolysis and GLUT1 localization in EGFR-mutated LAD cells. Moreover, our metabolomic analysis revealed that PI3K/AKT/mTOR signaling maintains de novo pyrimidine synthesis and the amino acid profile in EGFR-mutated LAD cells.

Whereas resting T cells have low metabolic requirements and mainly use oxidative phosphorylation to generate ATP, activated T cells shift metabolic activity to aerobic glycolysis in a manner similar to tumor cells (34,35). During T cell activation, several metabolic check points have been suggested to influence cell cycle, differentiation, cell fate and immunological function (34,35). A recent review theorized that metabolic check points may exist to determine cell fate even in cancer cells, since many factors which have been characterized as cell death regulators are known to modulate metabolic enzyme activity (36). Here we show that treatment of LAD cells with TKIs and PI3K/mTOR inhibitors diminish the levels of metabolites in glycolysis and PPP, although it is still not clear which metabolite or metabolic enzyme is responsible for LAD cell survival. Our results suggest that one metabolic checkpoint in EGFR-mutated LAD cells could be glucose metabolism.

The molecular mechanism by which PI3K/AKT/mTOR signaling regulates glucose transport is still unclear. In adipocytes and skeletal muscle, insulin signaling and the PI3K/AKT pathway stimulate the translocation of intracellular GLUT4 to the cell surface to promote glucose uptake into cells (37). The insulin signaling pathway is regulated through AS160 (Akt substrate of 160 kDa) and Tbc1Ds to modulate Rab GTPase, and through Rho GTPase TC10a to act on other targets (37). To test whether GLUT1 localization in EGFR-mutant LAD cells is regulated by a molecular mechanism similar to GLUT4, we examined phosphorylation of AS160 proteins under our
experimental conditions. Unexpectedly, phosphorylated AS160 protein was not changed at 6 hours after treatment with any tested inhibitors (data not shown). In addition, the role of intracellular GLUT1 is still unknown. Thus, further investigation would be needed to characterize in greater detail the molecular mechanisms that control GLUT expression, activity, and translocation.

A previous study showed that newly synthesized GLUT4 accumulates within insulin-responsive compartments, whereas GLUT1 biosynthetically traffics to the plasma membrane in adipocytes (38,39). After reaching the plasma membrane, GLUT1 undergoes endocytosis and localizes to recycling endosomes, where membrane proteins are transported for recycling back to the plasma membrane (40,41). In this study, when EGFR and PI3K/akt/mTOR signaling were inhibited, GLUT1 was predominantly found at intracellular compartments. Our data raise the possibility that PI3K/akt/mTOR signaling plays an essential role in trafficking of GLUT1 from recycling endosomes and/or retention of GLUT1 at the plasma membrane. Further study will be required to understand the mechanism by which the PI3K/akt/mTOR signaling controls the intracellular dynamics of GLUT1.

A research report showed that tumor-associated mutant p53 (mutp53) stimulated the Warburg effect in cancer cells as a new mutp53 gain of function (42). Mup53 did not change the expression of GLUT1, but promoted aerobic glycolysis by inducing GLUT1 translocation to the plasma membrane, which was mediated by activated RAS homolog gene family member A (RHOA) and the downstream effector, Rho-associated, coiled-coil containing protein kinase 1 (ROCK). In our study, all three LAD cell lines possess homozygous mutp53 in that p.V218delV was identified in HCC827, p.R248Q in PC-9 and p.R273H in H1975 cells as shown in public databases (http://cancer.sanger.ac.uk/cosmic/ or http://dbtss.hgc.jp/). A possible molecular mechanism is that either PI3K/akt/mTOR signaling may regulate GLUT translocation by directly activating the RHOA/ROCK pathway or stimulate glycolysis through this mutp53 pathway. Another interesting study showed that 5'-AMP-activated protein kinase (AMPK)-dependent degradation of thioredoxin-interacting protein (TXNIP) upon metabolic stress led to enhanced glucose uptake via GLUT1 (43). The PI3K/akt/mTOR signaling pathway in EGFR-mutant LAD cells may be linked with cellular redox status, since the pentose phosphate pathway, which is a major source of nicotinamide adenine dinucleotide phosphate (NADPH) in animal cells, was down-regulated after PI3K/mTOR inhibition. The molecular linkages between PI3K/akt/mTOR signaling, oxidative stress, and GLUT1 are still unclear. Lee and colleagues recently demonstrated that GLUT1 was phosphorylated on S226 by protein kinase C (PKC) (44). This phosphorylation of S226 was required for the rapid increase in glucose uptake and enhanced GLUT1 localization to cell surface in endothelial cells. Additional experiments would be required to determine whether PI3K/akt/mTOR signaling controls glucose transport through the TXNIP, mutp53 or PKC pathway.

The application of metabolomics in oncology has focused on advances in both cancer imaging and therapy (15,45,46). A high rate of $^{18}$F-fluorodeoxyglucose (FDG) uptake on positron emission tomography (PET) was significantly associated with poor prognosis in lung cancer (47). From our work, we found that hyperactive glycolysis markedly increased lactate accumulation in lung tumor tissues (48). These results imply that targeting the PI3K/akt/mTOR – GLUT axis could be a feasible chemotherapeutic option to control tumor progression. Indeed, compounds inhibiting the PI3K/akt/mTOR pathway are currently in various stages of clinical development in oncology, however, the administration of inhibitors to the PI3K/akt/mTOR pathway has been associated with metabolic adverse events including hyperlipidemia and hyperglycemia (49-51). We showed that knock-down of GLUT1 decreased cell growth in EGFR-mutant LAD cells but did not induce complete cell death. This may be due to the redundancy of other glucose transporters such as GLUT3. Since the PI3K/akt/mTOR pathway regulates many downstream effectors by cross-talking with various compensatory signaling pathways (51), the cell death induced by inhibition of PI3K/akt/mTOR may be associated with several molecular mechanisms including the internalization and degradation of GLUT1. Although agents directly inhibiting GLUT1 are in early-phase evaluations, preclinical studies have demonstrated that GLUT1 inhibitors led to diminished tumor growth in vitro and in vivo (52). Given that PI3K/mTOR inhibitors repress aerobic glycolysis in EGFR-mutated LAD cells, further studies are warranted in order to understand how cancer cell
metabolism is regulated and to develop more effective therapeutic agents specifically targeted to the metabolic pathways that can limit cancer growth.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

HM and KT conceived and coordinated the study and wrote the paper. MT, SM and ES designed, performed and analyzed the experiments shown in Figures 1, 2 and 3. HM designed, performed and analyzed the experiments shown in Figures 4. KS, SU, YO and GI designed, performed and analyzed the experiments shown in Figures 5 and 6. MT designed, performed and analyzed the experiments shown in Figure 7. AO, RA, KG and HE contributed to the coordination, interpretation and analysis of data in this study. All authors reviewed the results and approved the final version of the manuscript.

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FOOTNOTES
The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; EGFR, epidermal growth factor receptor; LAD, lung adenocarcinoma; TKI, tyrosine kinase inhibitor; IC50, half maximal inhibitory concentration; PPP, pentose phosphate pathway; ECAR, extracellular acidification rate; OCR, oxygen consumption rate; GLUT, glucose transporter; CAD, carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase.

FIGURE LEGENDS
FIGURE 1. EGFR-mutant LAD cells are more sensitive to dual PI3K/mTOR inhibitors than MEK inhibitor. Cells were treated with inhibitors at the indicated concentrations for 72 h, and viability was assessed using the WST-8 assay. (A) HCC827, (B) PC-9 and (C and D) H1975 cells are shown. The data are shown as the mean ± standard deviation (S.D.) (n = 6). (A-C) Blue line: Erlotinib; Red line: AZD6244; Green line: BEZ235; (D) Magenta line: PKI-587; Yellow line: AZD9291. The in vitro half maximal inhibitory concentration (IC50) for the growth of HCC827 was determined to be 0.010 μM to erlotinib, >10 μM to AZD6244, 0.011 μM to BEZ235. PC-9 had an IC50 of 0.019 μM to erlotinib, >10 μM to AZD6244, and 0.005 μM to BEZ235. H1975 had an IC50 of >10 μM to erlotinib, >10 μM to AZD6244, 0.042 μM to BEZ235, 0.050 μM to AZD9291 and 0.042 μM to PKI-587.

FIGURE 2. Altered phosphorylation of EGFR signaling proteins in EGFR-mutant LAD cells after treatment with inhibitors. Western blot analysis showing phospho-EGFR (p-EGFR), total EGFR, phospho-ERK (p-ERK), total ERK, phospho-AKT (p-AKT), total AKT and β-actin as a loading control in HCC827, PC-9 and H1975 cells treated with the indicated inhibitors. Equivalent amounts of proteins from whole cell lysates were subjected to WB analysis to detect the indicated proteins.

FIGURE 3. Glycolytic activities decreased after inhibition of PI3K/AKT/mTOR, but not RAS/MEK/MAPK, pathway in EGFR-mutant LAD cells. (A) Cell growth responses at 6 h to 1 μM of indicated inhibitors were measured by Trypan Blue staining. The cell numbers for HCC827, PC-9, and H1975 cells treated with of DMSO (black), erlotinib (ERLO, red), AZD6244 (blue) BEZ235 (green), AZD9291 (yellow) and PKI-587 (magenta) were shown. The data are shown as the mean ± S.D. (n = 4). (B) Extracellular lactate production in HCC827, PC-9 and H1975 cell lines treated with DMSO (black), erlotinib (ERLO, red), AZD6244 (blue), BEZ235 (green), AZD9291 (yellow) and PKI-587 (magenta) at 6 h post-inhibitors treatment. Error bars indicate ± S.D. (n = 4-12). * p < 0.05; **, p < 0.01 versus control by two-tailed Student’s t test. (C) ECAR values of HCC827, PC-9 and H1975 cell lines treated with DMSO (black), erlotinib (ERLO, red), AZD6244 (blue), BEZ235 (green), AZD9291 (yellow) and PKI-587 (magenta) at 36 min of flux assay. All cells were treated with the indicated inhibitors (1 μM) for 6 h before each assay. Results were reported as the mean ± S.D. (n = 7-12). *, p < 0.05; **, p < 0.01 versus control by two-tailed Student’s t test.

FIGURE 4. Metabolomic profiling after inhibition of PI3K/AKT/mTOR pathways. Intracellular concentration (pmol/million cells) of key metabolites involved in glycolysis and pentose phosphate pathway (PPP) after the inhibition of EGFR signaling is shown. Error bars indicate ± S.D. (n = 3). Total metabolites were extracted from methanol from HCC827, PC9 or H1975 cells treated with DMSO (red) or inhibitors (blue or green) for 6 h. Representative metabolites were measured by electrospray ionization-mass spectrometry (ESI-MS). Total metabolites were quantitatively analyzed by capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS).
FIGURE 5. PI3K/AKT/mTOR signaling maintains membrane localization of GLUT1. (A) Representative images from Immunohistochemistry using GLUT1 antibody to identify cytosolic and membrane staining are shown. Scale bars indicate 20 μm. (B) Pie chart summarizes the percentages of EGFR-mutant LAD tissue sections that are negative for GLUT1 protein (blue), or expressing GLUT1 predominantly in the cytoplasm (red) or plasma membrane (green). (C) HCC827 cells stained with Alexa 488 (green, GLUT1), Alexa 594 (red, Na/K ATPase), and DAPI (blue, nuclei). Na/K ATPase was a positive control as a plasma membrane protein marker. Scale bars indicate 10 μm.

FIGURE 6. Inhibition of PI3K/AKT/mTOR pathway does not affect total GLUT1 protein expression but alters membrane-bound GLUT1 levels in EGFR-mutant LAD cells. (A) Western blot analysis showing GLUT1 and β-actin as a loading control in HCC827, PC-9 and H1975 cells treated with the indicated inhibitors. Equivalent amounts of proteins from whole cell lysates were subjected to WB analysis to detect total GLUT1 proteins. For flow cytometric analysis, LAD cells were treated with erlotinib (ERLO, 1 μM), BEZ235 (BEZ, 1 μM) or DMSO as a control for 6 hours. After fixation, cells were stained with a rabbit anti-GLUT1 antibody and FITC-conjugated anti-rabbit secondary antibody. Representative flow cytometry plots of GLUT1 expression in HCC827 (B), PC-9 (C) and H1975 (D) cells treated with DMSO or BEZ235. The mean fluorescence intensity for GLUT1 is shown for (E) HCC827, (F) PC-9 and (G) H1975 cells. Blue bars show background fluorescence with the IgG isotype control while red bars indicate fluorescence staining results with anti-GLUT1 Ab. Error bars indicate ± S.D. (n = 3). *P<0.05 versus control by two-tailed Student’s t-test.

FIGURE 7. Loss of GLUT1 in EGFR-mutant LAD cells decreases cellular proliferation and lactate production. (A) Western blot analysis of siGLUT1-treated LAD cells. siRNA targeting GLUT1 (SLC2A1) successfully knock-downed GLUT1 protein in PC-9 and H1975 cells as confirmed by WB. The size signal for GLUT1 was widely distributed from 37 to 150 kDa. β-actin was used as loading control for protein level. (B) Extracellular lactate production in PC-9 and H1975 cell lines transfected with siNC (red), siGLUT1#1 (blue) and siGLUT1#2 (green). (C and D) Involvement of GLUT1 in cell growth. PC-9 (C) and H1975 (D) cells transfected with GLUT1 siRNAs were incubated for the indicated times. The data are shown as the mean ± S.D. (n = 3). *P<0.05, **P<0.01 (Student’s t-test).
Makinoshima, et al., FIGURE 1
|              | HCC827 |              | PC-9 |              | H1975 |
|--------------|--------|--------------|------|--------------|-------|
|              | DMSO   | Erlotinib    | AZD6244 | BEZ235       | DMSO  | Erlotinib | AZD6244 | BEZ235       | DMSO  | AZD9291 | DMSO  | PKI-587 |
| p-EGFR       | ![Image] | ![Image]     | ![Image] | ![Image]     | ![Image] | ![Image] | ![Image] | ![Image]     | ![Image] | ![Image] | ![Image] | ![Image] |
| EGFR         | ![Image] | ![Image]     | ![Image] | ![Image]     | ![Image] | ![Image] | ![Image] | ![Image]     | ![Image] | ![Image] | ![Image] | ![Image] |
| p-ERK1/2     | ![Image] | ![Image]     | ![Image] | ![Image]     | ![Image] | ![Image] | ![Image] | ![Image]     | ![Image] | ![Image] | ![Image] | ![Image] |
| ERK1/2       | ![Image] | ![Image]     | ![Image] | ![Image]     | ![Image] | ![Image] | ![Image] | ![Image]     | ![Image] | ![Image] | ![Image] | ![Image] |
| p-AKT        | ![Image] | ![Image]     | ![Image] | ![Image]     | ![Image] | ![Image] | ![Image] | ![Image]     | ![Image] | ![Image] | ![Image] | ![Image] |
| AKT          | ![Image] | ![Image]     | ![Image] | ![Image]     | ![Image] | ![Image] | ![Image] | ![Image]     | ![Image] | ![Image] | ![Image] | ![Image] |
| β-actin      | ![Image] | ![Image]     | ![Image] | ![Image]     | ![Image] | ![Image] | ![Image] | ![Image]     | ![Image] | ![Image] | ![Image] | ![Image] |

Makinoshima, et. al., FIGURE 2
Makinoshima, et. al., FIGURE 3
Makinoshima, et. al., FIGURE 4
Figure 5

Makinoshima, et. al., FIGURE 5
Makinoshima, et al., FIGURE 6
Makinoshima, et. al., FIGURE 7
Signaling through the Phosphatidylinositol 3-Kinase (PI3K)/Mammalian Target of Rapamycin (mTOR) Axis is Responsible for Aerobic Glycolysis mediated by Glucose Transporter in Epidermal Growth Factor Receptor (EGFR)-mutated Lung Adenocarcinoma

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