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mTORC2 contributes to the metabolic reprogramming in EGFR tyrosine-kinase inhibitor resistant cells in non-small cell lung cancer

Chun-Te Chiang, **Alexandra N. Demetriou**, **Nolan Ung**, **Niharika Choudhury**, **Kimya Ghaffarian**, **Daniel L. Ruderman**, **Shannon M. Mumenthaler***

Lawrence J. Ellison Institute for Transformative Medicine of USC, University of Southern California, Los Angeles, CA, USA

Abstract

Non-small cell lung cancer (NSCLC) patients with activating *EGFR* mutations are often successfully treated with EGFR tyrosine kinase inhibitor (TKI) such as erlotinib; however, treatment resistance inevitably occurs. Given tumor metabolism of glucose and therapeutic response are intimately linked, we explored the metabolic differences between isogenic erlotinib-sensitive and -resistant NSCLC cell lines. We discovered that the growth of erlotinib-resistant cells is more sensitive to glucose deprivation. Seahorse metabolic assay revealed erlotinib-resistant cells have lower spare respiratory capacity (SRC), an indicator of metabolic flexibility, compared to erlotinib-sensitive cells. Additionally, we found downstream components of mTORC2 signaling to be phosphorylated in erlotinib-resistant cells. Knockdown of an mTORC2 component, Rictor, enhanced the SRC and rescued the growth rate of erlotinib-resistant cells during glucose deprivation. Among NSCLCs with activating *EGFR* mutations, gene sets involved in glucose metabolism were enriched in patients with high expression of p-NDGR1, a readout of mTORC2 activity. Furthermore, overall survival was negatively correlated with p-NDRG1. Our work uncovers a link between mTORC2 and metabolic reprogramming in EGFR TKI-resistant cells and highlights the significance of mTORC2 in the progression of *EGFR*-mutated NSCLC.

Keywords

NSCLC; EGFR TKI resistance; Glucose metabolism; mTORC2

*Corresponding author. Department of Medicine, University of Southern California, 2250 Alcazar Street, CSC 242, Los Angeles, CA, 90033, USA. smumenth@usc.edu (S.M. Mumenthaler).

Author contributions

C.T.C., A.N.D. and K.G. conducted experiments. N.U. analyzed TCGA database. D.L.R. and N. C. performed the statistical analyses. C.T.C. and S.M.M. wrote the manuscript and conceptualized the framework for this research. All authors helped edit the manuscript.

Conflicts of interest

The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.canlet.2018.07.025.

1. Introduction

Lung cancer is the leading cause of cancer-related death globally [1]. Non-small cell lung cancer (NSCLC) comprises 85% of diagnosed lung cancer cases and has a grim 5-year survival rate of 18%. Approximately 15% of U.S. and 40% of Asian NSCLC cases harbor mutations in the EGFR kinase domain which confer sensitivity to small-molecule EGFR tyrosine-kinase inhibitors (TKIs) such as erlotinib [2]. However, in a majority of cases, treatment resistance emerges within 1 year despite robust initial response rates [3]. Several genetic mechanisms of resistance to EGFR TKIs have been observed clinically and include a secondary *EGFR* mutation (T790 M) and *MET* amplification [4]. Despite a new generation of EGFR TKIs approved to treat patients with *EGFR* T790 M, studies have shown that cancer cells can still develop resistance [5–7]. To improve the outcomes in patients with *EGFR*-mutant NSCLC, it is critical to understand the molecular escape mechanisms to EGFR inhibition.

Altered tumor cell metabolism is a hallmark of human cancer and considered to be a therapeutic target for treating cancer [8]. Oncogenic pathways have been linked to the rewiring of tumor metabolism to support cellular energetics and supply macromolecule precursors in cancer. Several studies have shown that NSCLC displays a higher rate of glucose metabolism compared to normal lung tissue [9,10]; therefore, there is significant interest in identifying metabolic vulnerabilities that can be exploited as new therapeutics. In particular, it has been shown that EGFR signaling regulates glucose metabolism in NSCLC cells through the PI3K/mTOR pathway [11,12]. mTOR is a crucial regulator of cell growth, proliferation and metabolism [13], and mTOR activity is also involved in the development and progression of *EGFR* mutated lung tumors [14]. These results prompted us to explore glucose utilization and the mTOR pathway in connection with resistance to EGFR TKIs.

To examine the behavior of EGFR TKI-sensitive and -resistant cells in response to environmental perturbations, we previously developed a high-content imaging workflow to dynamically phenotype cells in various microenvironmental contexts [15,16]. We used this workflow to investigate the impact of glucose deprivation on cell behavior and identified differential growth kinetics between erlotinib-sensitive and -resistant isogenic NSCLC cells. Given the significant influence of mitochondrial function on the response to glucose deprivation [17], we utilized a Seahorse metabolic assay to interrogate the spare respiratory capacity (SRC) of the NSCLC cells. SRC is the extra mitochondrial capacity available for cells to use in response to stress or increased ATP demand [18] and it has been implicated in the ability of cells to cope with oxidative metabolic stress [19]. We demonstrated erlotinib-resistant cells have less SRC compared to erlotinib-sensitive cells. Interestingly, we found that the activity of mTOR2, but not mTORC1, was increased in erlotinib-resistant cells and may contribute to the diminished SRC observed in erlotinib-resistant cells. Data from The Cancer Genome Atlas (TCGA) Research Network revealed a correlation between mTORC2 signaling and a shorter overall survival time in patients with *EGFR*-mutated NSCLC. These results indicate that EGFR TKI-resistant cells have distinct metabolic programs that may be exploited to predict tumor progression and stratify patients with *EGFR*-mutated NSCLC for better treatment outcomes.

2. Material and methods

2.1. Cell culture

All NSCLC cell lines used in this study carry *EGFR*-activating mutations in either exon 19 or exon 21. The isogenic erlotinib-sensitive and -resistant (R) cell lines (PC9, and PC9R-T790 M; H3255 and H3255R-T790 M; HCC4011, HCC4011R-*MET* amplified) were originally acquired from Dr. William Pao (while at Vanderbilt University) and cultured in RPMI 1640 media. Erlotinib-resistant cell lines were maintained in 1 μM erlotinib as previously described [23]. All cell lines were regularly tested for mycoplasma contamination using MycoAlert (Lonza #LT07-518) and authenticated by professional authentication services (University of Arizona Genetic Core).

2.2. Reagents

Erlotinib (#S1023) was obtained from Selleck Chemicals (Houston, TX, USA). Puromycin (#A1113803) was purchased from Life Technologies (Carlsbad, CA, USA). Hoechst 33342 (#H21492) and propidium iodine (#P1304MP) were acquired from Invitrogen (Waltham, MA, USA). The Rictor (#2114), phospho-tyrosine (#5465), phospho-NDRG1 (T346; #5482), NDRG1 (#9485), phospho-Akt (S473; #4060), Akt (#9272), phospho-S6 (S240/4; #2215), S6 (#2217), phospho-4EBPl (T37/46; #2855), 4EBP1 (#9452), phospho-EGFR (Y1068; #3777), EGFR (#4267) and MET (#8198) antibodies were from Cell Signaling Technologies (Danvers, MA, USA). Antibodies against β-actin (#A1978) were purchased from Sigma (St. Louis, MO, USA).

2.3. High-content screening and image analysis

The effect of environmental perturbation on cell growth was measured as described previously [15]. In brief, cells were seeded in 96 well CellCarrier plates (PerkinElmer #6005550). 1 day after seeding, cells were treated with the indicated environmental perturbations. Each condition was assayed in at least triplicate wells. Prior to imaging using the Operetta® High-Content Screening System (PerkinElmer #HH12000000), cells were stained with 5 μg/ml of Hoechst 33342 and 5 μg/ml of propidium iodine for 30 min to identify live or dead cells, respectively. Image analysis was performed using the Harmony 3.5.2 software (PerkinElmer #HH17000001).

2.4. Growth rate calculation

The measured live and dead cell counts at various time points under glucose depletion or repletion conditions were fit to an exponential growth model [20]. A linear regression of the log-transformed data was performed to obtain fitted rates for each condition.

2.5. Agilent seahorse XF metabolic assays

Metabolic analysis of erlotinib-sensitive and -resistant were measured using a Seahorse XFp Mito Stress Test (Agilent #103010-100). In brief, 10,000 cells were seeded in each well of an XFp cell culture plate. At day 2, real-time oxygen consumption and proton production rates were determined, and all the values were normalized to protein concentration using a standard BCA protein assay.

2.6. Western blot

Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer (Sigma #R0278) supplemented with halt protease and phosphatase inhibitor cocktail (Thermo Scientific # 78442). Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific #23223) per the manufacturer's instructions. Equal amounts of protein extracts were separated using 4–15% mini-Protean TGX polyacrylamide gel (Bio-Rad #456–1033). Samples were transferred to a Trans-Blot Turbo PVDF membrane (Bio-Rad #1704156). The membrane was blocked using Blotting-Grade Blocker (Bio-Rad #170–6404) dissolved in TBST and probed with varying primary antibodies. The membrane was then treated with HRP conjugated secondary antibodies. The immunoreactivity was detected with either the Pierce ECL Western Blotting Substrate or the Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific). Densitometric analysis was performed with ImageJ, and relative values were displayed under respective blots.

2.7. shRNA transfection

Rictor shRNA was a gift from David Sabatini (Addgene plasmid # 1853) [21]. Control shRNA was purchased from Santa Cruz (# 108080). Lentivirus-mediated delivery of shRNA was performed 24 h after passaging in the presence of 5 μg/ml polybrene (Santa Cruz # 134220). Cells were selected and maintained in 1 μg/ml puromycin.

2.8. Mining of public datasets

Mutation, mRNA and protein expression data were all downloaded from The Cancer Genome Atlas (TCGA) Research Network (http://cancergenome.nih.gov/). Patients within the luad_tcga cohort (lung adenocarcinoma TCGA provisional) with mutant EGFR-driven lung adenocarcinoma and accompanying reverse phase protein array (RPPA) and RNAseq data were selected for analysis (Supplementary Table 1). Relative protein expression data were obtained from the level 4 RPPA data. Data were normalized and batch corrected accordingly [22]. RNAseq profiles were analyzed using the GSEA 3.0 desktop tool. Patients were divided into two subsets, those with p-NDRG1 expression below the cohort median and those above. The log2fold change was calculated using DEseq2 and used as input to the pre-ranked algorithm of the GSEA tool using the classic enrichment statistic as described http://www.broadinstitute.org/gsea. The Hallmark_Glycolysis and _Oxidative Phosphorylation gene sets from the Broad Institute's MsigDB (http://software.broadinstitute.org/gsea/msigdb/index.jsp) were used for gene set enrichment analysis. The normalized enrichment score (NES) and false discovery rate (FDR) were calculated for comparison. Disease-free survival and overall survival clinical data were obtained from TCGA using the cdgsr package in the R statistical programming language.

2.9. Statistical analysis

All values were expressed as mean ± SEM. Studen's *t*-test (two-tailed) was performed for statistical comparison. Asterisk indicates that the values are significantly different (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

3. Results

3.1. Doubling time increased under glucose deprivation in EGFR TKI-Resistant cells

For these studies, we used three human NSCLC isogenic erlotinib-sensitive and -resistant (R) cell lines (PC9, PC9R; H3255, H3255R;HCC4011, and HCC4011R). PC9 exhibit exon 19 in-frame deletions (delE746-A750). H3255 and HCC4011 have exon 21 L858R mutations. PC9R and H3255R contain an additional *EGFR* mutation T790 M. HCC4011R has *MET* amplification. The growth of PC9R, H3255R, and HCC4011R was not affected under 1 μM Erlotinib treatment (Fig. S1A). T790 M mutation was validated through sequencing of EGFR exon 20 (Fig. S1B). Consistent with previous observations, PC9R cells showed further EGFR amplification, and HCC4011R cells exhibit an increase in MET expression while decreasing EGFR expression (Fig. S1C) [23].

Traditional cell growth assays (e.g. MTT) often use metabolic activity as a surrogate measurement of cell number. Although these assays are routinely employed, they may fail to provide an accurate assessment of cell behavior under certain conditions [24]. Therefore, to determine cellular responses to environmental perturbations, we previously developed a high-content image-based workflow for quantitatively assessing cell dynamics over time [15]. We used this workflow to investigate the effects of glucose deprivation on the cell growth kinetics of the three erlotinib-sensitive and -resistant isogenic NSCLC cells. In general, we observed erlotinib-resistant cells grew more slowly in glucose depleted conditions compared to erlotinib-sensitive cells (Fig. S2). To quantify the effects of glucose deprivation and avoid time-dependent bias, we fit the cell counts collected at different time points into a mathematical model of exponential growth using CellPD (cell phenotype digitizer) [20]. As shown in Fig. 1, erlotinib-resistant cells exhibited a greater increase in doubling time under glucose deprivation compared with isogenic erlotinib-sensitive cells. The relative doubling time increase under glucose deprivation in PC9 vs. PC9R, H3255 vs. H3255R, and HCC4011 vs. HCC4011R were 113% vs. 134%, 222% vs. 273%, 130% vs. 160%, respectively. This effect was independent of any growth rate differences under glucose repletion given the PC9 cells grew faster than PC9R cells while H3255 and HCC4011 grew slower than H3255R and HCC4011R cells, respectively. These results indicate that the growth of erlotinib-resistant cells was more sensitive to glucose deprivation.

3.2. Erlotinib-resistant cells have lower spare respiratory capacity

Mitochondrial function has been shown to play a pivotal role in the response to glucose deprivation [17]. To determine whether the differential growth kinetics of erlotinib-sensitive and -resistant cells under glucose deprivation are due to differing mitochondrial functions between these cell types, we utilized the Seahorse extracellular flux analyzer platform to interrogate the mitochondrial metabolic phenotypes of the isogenic cell lines. Through the Cell Mito Stress Test, the ATP-linked oxygen consumption rate (OCR) and SRC can be calculated after sequential injections of ATP synthase inhibitor oligomycin, protonophoric uncoupler FCCP and electron transport inhibitors rotenone plus antimycin A. The ATP-linked OCR was calculated by taking the difference between basal OCR and OCR following addition of oligomycin while the SRC was determined by taking the difference between basal OCR and OCR following addition of FCCP. We found PC9R cells had a 22% decrease

in ATP-linked OCR and a 51% diminished SRC compared to the PC9 cells (Fig. 2A and B). We observed a 31% and 63% decrease in SRC in H3255R cells and HCC4011R when compared to the isogenic H3255 and H4011 cells, respectively (Fig. 2C and D). SRC is an indicator of cell metabolic flexibility. Specifically, it is the extra mitochondrial capacity available in a cell to produce energy in response to an increase in energy demand. Previous work has shown that diminished SRC can impede a cell's ability to cope with metabolic stress [19]. Our results indicate that the lower SRC in erlotinib-resistant cells rendered them more susceptible to glucose deprivation.

3.3. mTORC2 contributes to the metabolic reprogramming in erlotinib-resistant cells

Mutant EGFR has been shown to regulate glucose metabolism through activation of PI3K/mTOR pathway in NSCLC cells [11]. mTOR is a serine/threonine kinase and exists as two distinct complexes, known as mTORC1 and mTORC2. mTORC1 promotes the translation of genes involved in protein, nucleotide, and lipid synthesis while mTORC2 is thought to be implicated in cell survival and proliferation [13]. Recent reports demonstrate that mTORC2 activation can drive tumor progression and therapeutic resistance in brain and breast cancers [25,26]. These findings led us to address the role of mTORC1 and mTORC2 in the metabolic reprogramming of erlotinib-resistant cells.

We examined the phosphorylation of ribosomal protein S6 and 4E-binding protein 1 (4E-BP1) as mTORC1 activity readout and measured the phosphorylation of Akt and N-Myc downstream-regulated gene 1 (NDRG1) as a surrogate for mTORC2 activity. S6 is phosphorylated by S6K, which is a direct substrate of mTORC1 [27]. 4E-BP1 is regulated by mTORC1 and is involved in cap-dependent translation [28]. NDRG1 is phosphorylated by serum and glucocorticoid-inducible kinase (SGK), which is a direct substrate of mTORC2 [29]. Akt is regulated by mTORC2 and promotes cell growth and survival [30]. No increase in mTORC1 activity was noted between erlotinib-sensitive and -resistant cells (Fig. 3A). In contrast, an increase in phosphorylation of NDRG1 and Akt was observed in all the isogenic erlotinib-resistant cells compared to erlotinib sensitive-cells (Fig. 3A). These results reveal mTORC2 activation in erlotinib-resistant cells.

While mTORC1 and mTORC2 share some common components, Rictor is a subunit specific to mTORC2 [30]. To test whether mTORC2 activation contributes to the metabolic reprograming in erlotinib-resistant cells, we used a genetic approach to knockdown Rictor expression. After transducing PC9 and PC9R cells with lentivirus encoding Rictor shRNA sequences (shRictor), or a scrambled control shRNA sequence (shCon), cells expressing shRictor displayed downregulation of Rictor protein and decreased phosphorylation of NDRG1 and Akt (Fig. 3B), indicating the inhibition of mTORC2 signaling. Additionally, we found Rictor knockdown in PC9R cells resulted in an increase in SRC (Fig. 3C) and decreased sensitivity to glucose deprivation compared to PC9R shCon (Fig. 3D and S3). It has been shown that glucose withdrawal induces supra-physiological phospho-tyrosine signaling that leads to cell death [31]. Consistent with these previous findings, we found phospho-tyrosine signaling was increased after glucose withdrawal in the erlotinib-resistant cells (Fig. 3E). Moreover, the downstream signaling of mTORC2, but not mTORC1, was increased following glucose withdrawal. Notably, Rictor knockdown was able to reduce the

glucose-deprived induction of mTORC2 activation (Fig. 3E). Our data collectively suggest mTORC2 activation is implicated in the metabolic reprogramming in erlotinib-resistant cells.

3.4. mTORC2 activation is associated with glucose metabolism and correlated with outcome of patients with EGFR-mutant NSCLC

To investigate the clinical implication of mTORC2 activation in patients with *EGFR*-mutated NSCLC, we analyzed publicly available data from TCGA to investigate the biological pathways associated with mTORC2 signaling (as measured by p-NDRG1 [32]). Based on the cohort median, we divided NSCLC patients with *EGFR* driver mutations into two groups, those with relatively high p-NDRG1 expression and those with relatively low expression. We performed gene set enrichment analysis (GSEA) to identify gene sets enriched between these two groups. We found genes involved in glycolysis were upregulated in the high p-NDRG1 expressing group (Normalized Enrichment Score = 3.360, FDR < 0.001) (Fig. 4A). In addition, a panel of oxidative phosphorylation genes were downregulated in this group (Normalized Enrichment Score = −2.961, FDR < 0.001) (Fig. 4B). These results link mTORC2 activation with glucose metabolism in *EGFR*-mutant NSCLC. Within the database, ten patients with follow-up survival data were used for further analysis. We found a significant negative correlation between p-NDRG1 expression and overall survival ($R = -0.0.67$, $p < 0.05$) (Fig. 4C), but no such association was observed for mTORC1 readouts (p-S6 and p-4EBP) (Fig. S4). In addition, we did not observe this negative correlation in patients with wild-type *EGFR* (Fig. S5), suggesting the association between p-NDRG1 and overall survival is mutant-EGFR dependent. A negative correlation was also found between p-NDRG1 expression and progression-free survival, although this is not statistically significant, as there are only 7 patients with progression-free survival data within the database ($R = —0.0.61$, $p = 0.08$; data not shown). These results suggest that the expression of p-NDRG1 may be an indicator of tumor progression based on glucose metabolism.

4. Discussion

The movement toward precision medicine for cancer treatment has been hindered by the emergence of drug resistance to targeted therapy. Although EGFR TKIs are the standard of care for patients with *EGFR*-mutant NSCLC, TKIs are not curative. Several resistance mechanisms to first-generation EGFR TKIs such as $EGFR^{T790M}$ and *MET* amplification have been identified and characterized. However, the mechanism underlying the evolution of resistance is still unclear. Some studies have suggested that resistant clones may be present before treatment and are rapidly selected out during TKI treatment [33–35]. Other studies suggest that resistance can emerge from drug-tolerant persister cells [33,36,37]. Accumulating evidence suggests that *EGFR* mutations can drive alterations in metabolism [12,38], which led us to hypothesize that metabolic reprogramming plays a pivotal role in the emergence of EGFR TKI resistance. In the present study, high-content imaging enabled us to interrogate and uncover a differential impact of glucose starvation on isogenic erlotinib-sensitive and -resistant cell growth. We further demonstrated that mTORC2 signaling contributes to the metabolic reprogramming in erlotinib-resistant cells.

Through TCGA analysis of human NSCLC specimens with mutant *EGFR*, we found a negative correlation between mTORC2 activity readout p-NDRG1 and patient survival. It is noteworthy that most of the patients did not carry known genetic mutations that cause resistance to EGFR TKIs; however, GSEA revealed that gene sets involved in glucose metabolism are altered between patients with high or low mTORC2 activity. A recent report also suggested that metabolic alterations such as lactate utilization can be correlated with NSCLC progression after several years of follow up [39]. These results not only indicate that metabolic reprogramming could happen before the emergence of genetic resistance mutations, but also suggest mTORC2 activation as a novel readout of metabolic changes that could be used to predict the progression of NSCLC patients with *EGFR*-activating mutations.

It is well known that mTORC1 controls cell size and growth by regulation of protein translation, while the function and regulation of mTORC2 are less clear and still being actively explored [13]. Studies have shown that mTORC2 can regulate cell survival and stress response by phosphorylating Akt and SGK. Akt integrates signals from PI3K and mTORC2 and is frequently activated in NSCLC to promote cell growth and survival. SGK phosphorylates NDRG1 and has been demonstrated to promote drug resistance [40,41]. We investigated the readouts of mTORC1 and mTORC2 in isogenic *EGFR* mutated NSCLC cells and found that downstream signaling components of mTORC2, but not mTORC1, are phosphorylated in erlotinib-resistant cells. Loss of mTORC2 signaling through knockdown of Rictor led to the finding that mTORC2 is involved in the metabolic reprogramming in erlotinib-resistant cells. Recent studies in glioblastoma also demonstrate a role for mTORC2 in controlling aerobic glycolysis and resistance to chemotherapy and targeted therapy [26,32,40,42]. Our results corroborate previous findings to suggest mTORC2 acts as a central node connecting glucose metabolism with therapeutic resistance.

Our experiments demonstrate that the metabolic reprogramming in erlotinib-resistant cells results in lower SRC to cope with glucose deprivation-mediated environmental stress. SRC is an indicator of mitochondria energetic status in cells; substrates such as pyruvate, amino acids, and fatty acids are thought to play a critical role in regulating SRC [18]. Pyruvate dehydrogenase converts pyruvate to acetyl-CoA for entry into the TCA cycle and oxidative phosphorylation [43]. Amino acids such as glutamate and aspartate can enter the TCA cycle through conversion to α-ketoglutarate and oxaloacetate [44]. Fatty acids can also enter TCA cycle through β-oxidation [45]. Whether mTORC2 regulates the substrate supply for mitochondria respiration in EGFR TKI-resistant cells is currently under investigation.

Overall, we provide evidence of metabolic reprogramming during EGFR TKI treatment mediated through mTORC2 signaling. Additionally, we found *EGFR*-mutated NSCLC patients with mTORC2 activation tend to have worse clinical outcomes. These findings suggest stratification of patients with mTORC2 activation could help optimize treatment plans to improve therapeutic outcomes for patients with activating *EGFR* mutation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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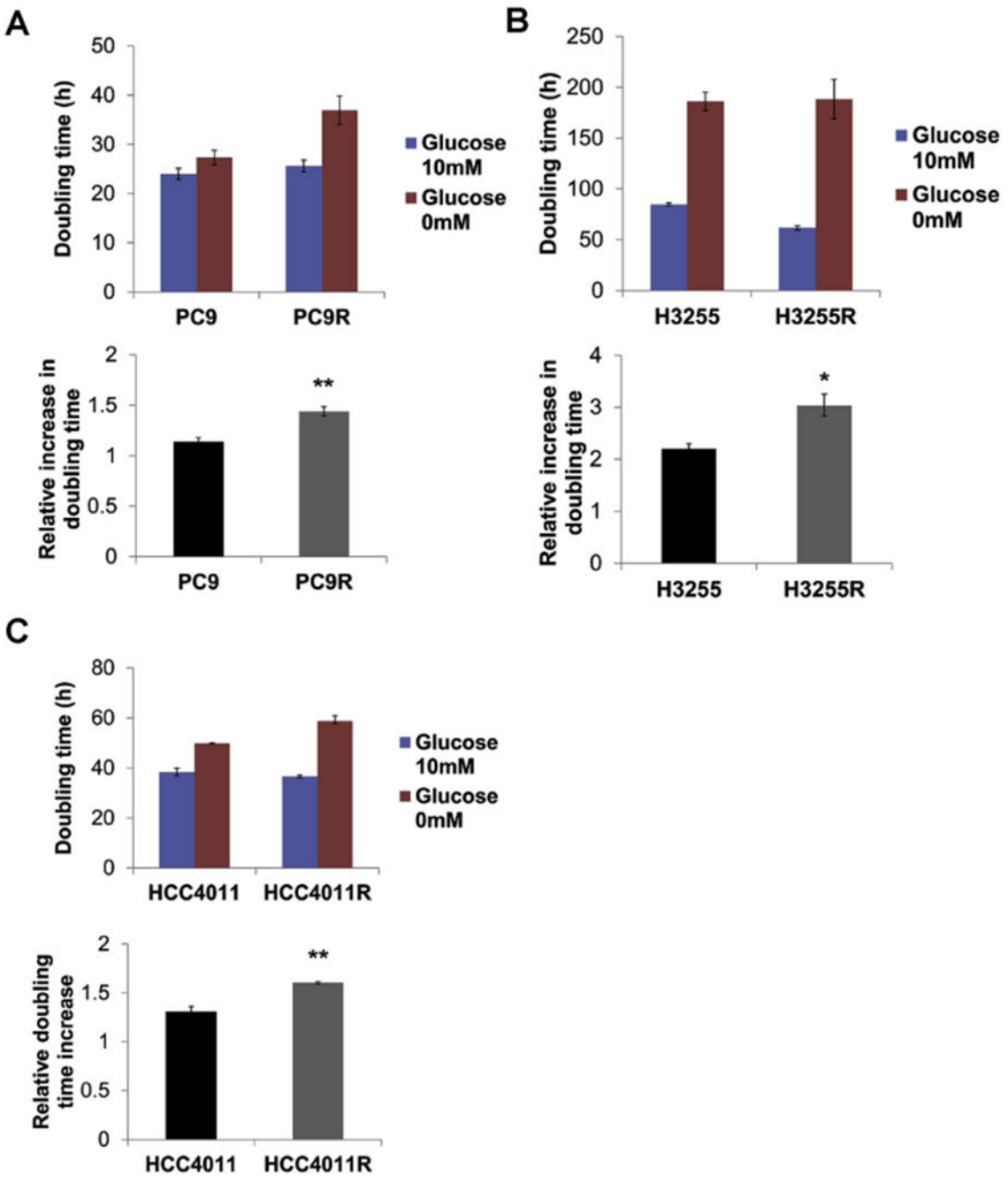

Fig. 1.

The effects of glucose deprivation on the growth of isogenic erlotinib-sensitive and -resistant cells. (A–C) *Top panel* PC9, PC9R, H3255, H3255R, HCC4011 and HCC4011R cells were plated in complete culture media and replaced with complete or glucose-free medium the following day. Cell counts were measured at several time points using Operetta® high-content screening platform. Doubling time was calculated by dividing the natural logarithm of 2 by the exponential growth rate. *Bottom panel*, relative doubling time under glucose

deprivation was normalized to the doubling time under complete medium. Data are average of three individual experiments. Bars, SEM. **P < 0.01, *P < 0.05.

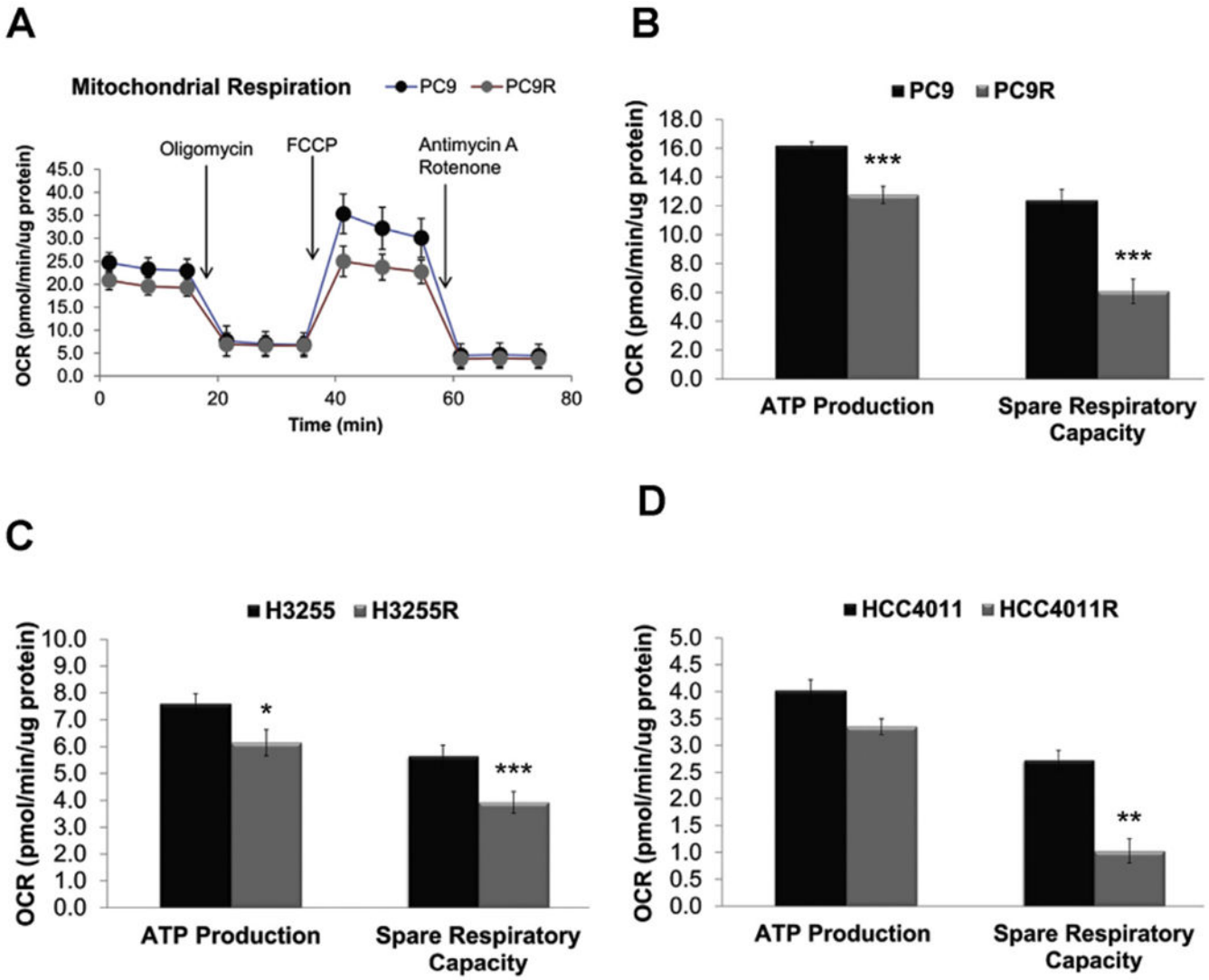

Fig. 2.

Erlotinib-resistant cells have lower spare respiratory capacity. (A) Mitochondrial stress test profiles for PC9 and PC9R cells. (B–D) Mitochondrial ATP linked oxygen consumption and mitochondrial reserve capacity of PC9, PC9R, H3255, H3255R, HCC4011 and HCC4011R cells. Data are average of three individual experiments. Bars, SEM. ***P < 0.001, **P < 0.01, *P < 0.05.

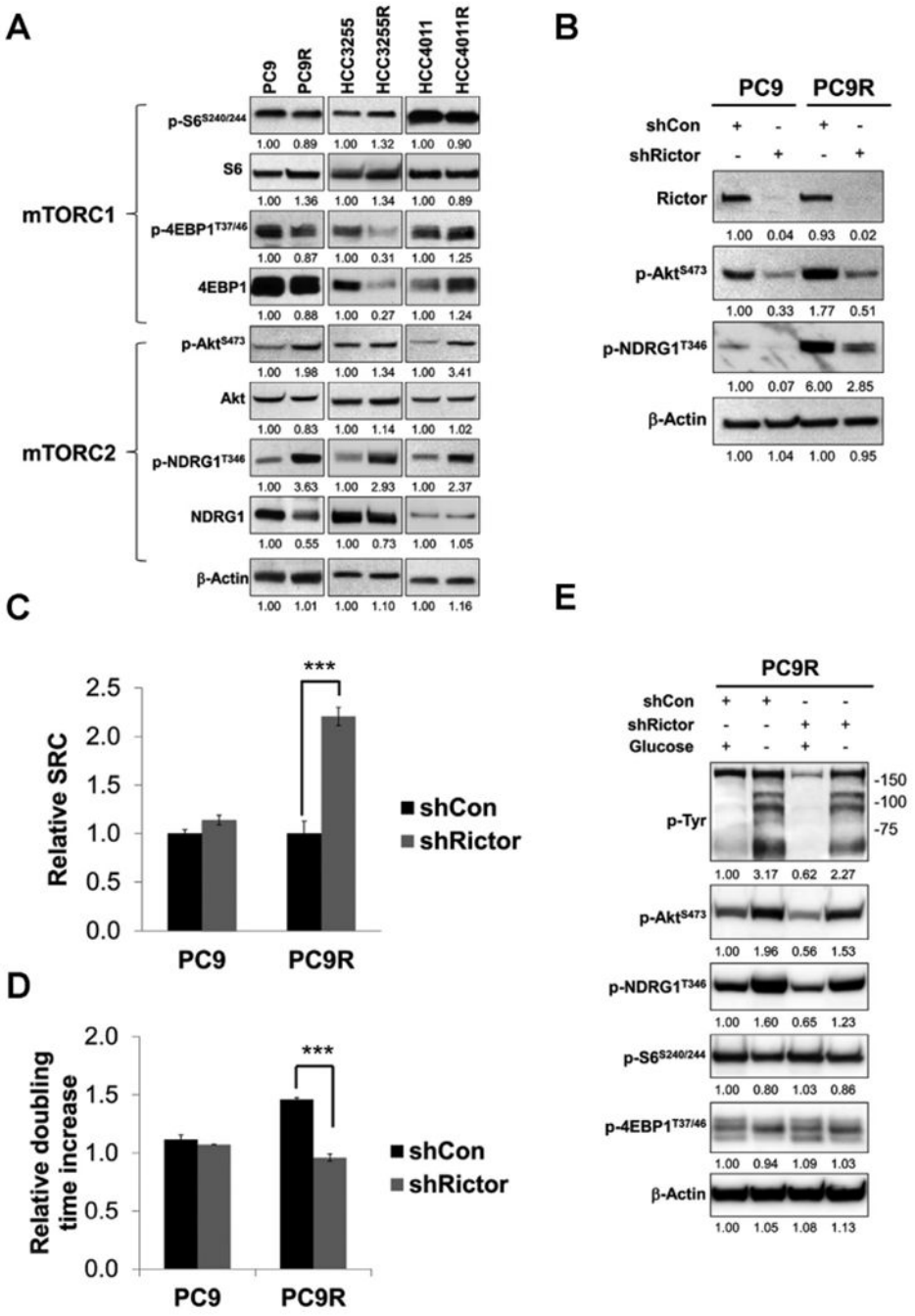

Fig. 3.

Inhibition of mTORC2 signaling increases spare respiratory capacity in erlotinib-resistant cells. A) Whole-cell lysates from isogenic NSCLC cell lines were subjected to western blotting analysis of mTORC1 and mTORC2 activities. *Values below the figures,* relative changes normalized to isogenic erlotinib-sensitive cells. (B) Whole-cell lysates from PC9 cells and PC9R cells stably transduced with the control shRNA (shCon) or Rictor shRNA (shRictor) were subjected to western blotting analysis of Rictor, p-Akt, p-NDRG1 and β-actin. *Values below the figures,* relative changes normalized to PC9 shCon. (C) The

relative spare repository capacity (SRC) of PC9 cells and PC9R cells stably transduced with the control shRNA (shCon) or Rictor shRNA (shRictor) were measured by Seahorse mito stress assay. (D) PC9 cells and PC9R cells stably transduced with the control shRNA (shCon) or Rictor shRNA (shRictor) were cultured in the presence or absence of glucose. The relative doubling time was calculated as described in Fig. 1. (E) PC9R cells stably transduced with the control shRNA (shCon) or Rictor shRNA (shRictor) were treated with complete or glucose-free medium for 24 h. Whole-cell lysates were subjected to western blotting analysis. *Values below the figures,* relative changes normalized to PC9R shCon in glucose repletion condition. Bars, SEM. ***P < 0.001.

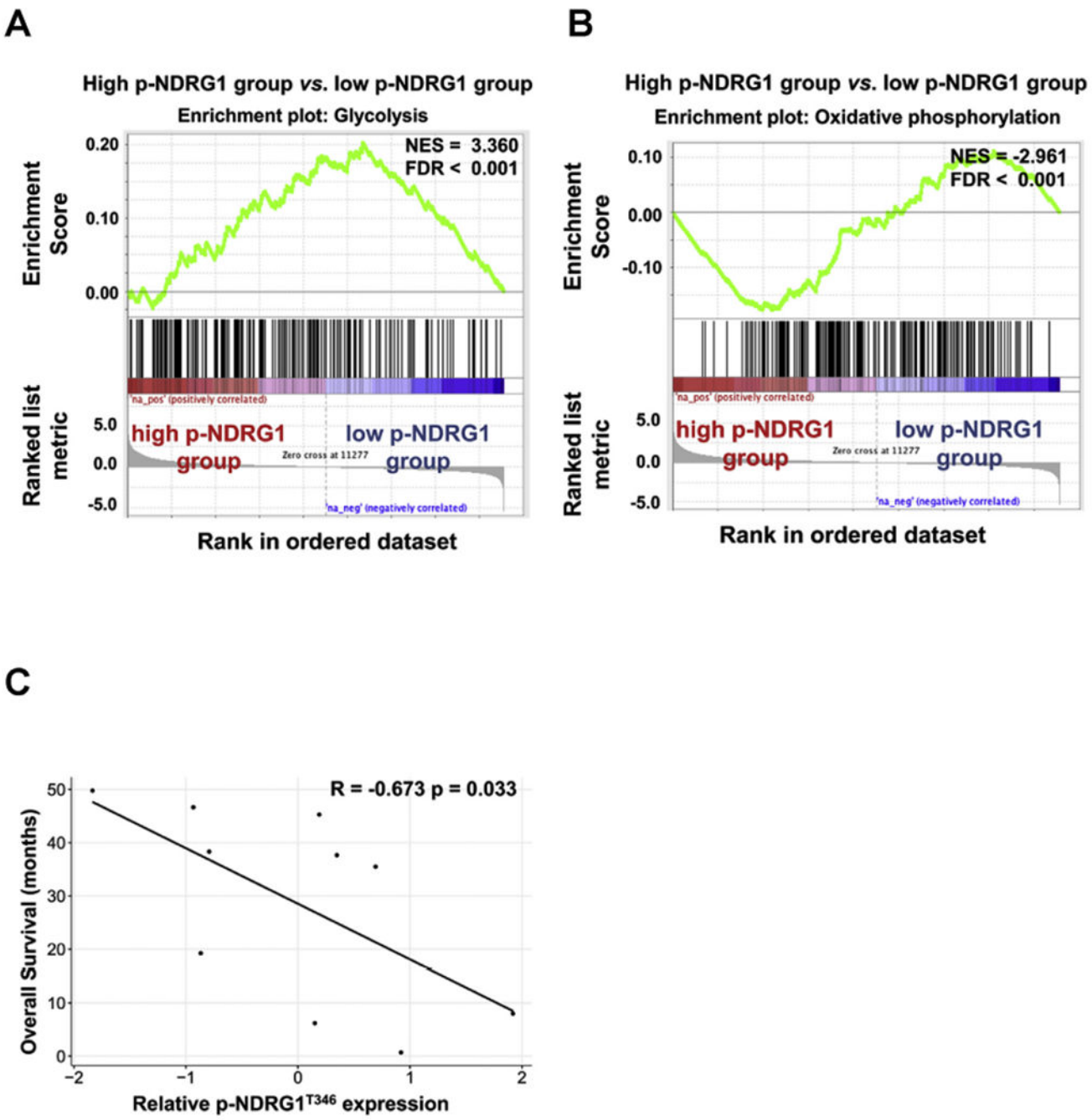

Fig. 4.

p-NDRG1 is able to predict the outcome of *EGFR*-mutant NSCLC patients. (A and B) GSEA revealed an enrichment of glycolytic genes in patients with *EGFR*-mutant NSCLC tumors with high p-NDRG1 expression (A) and an enrichment of oxidative phosphorylation genes in *EGFR*-mutant NSCLC patients with low p-NDRG1 expression (B). NES, normalized enrichment score. FDR, false discovery rate. (C) Correlation between p-NDRG1 expression and overall survival (OS) in NSCLC patients with *EGFR* driver mutations. The reverse phase protein array (RPPA) data for the relevant patients were

downloaded from The Cancer Proteome Atlas (TCGA) database. The X-axis shows batch-normalized protein expression levels of p-NDRG1. The survival time of each patient is shown in the Y-axis in months. Black line denotes the linear fit.