Metabolic Determinants of Sensitivity to Phosphatidylinositol 3-Kinase Pathway Inhibitor in Small-Cell Lung Carcinoma

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Abstract

Comprehensive genomic analysis has revealed that the PI3K/AKT/mTOR pathway is a feasible therapeutic target in small-cell lung carcinoma (SCLC). However, biomarkers to identify patients likely to benefit from inhibitors of this pathway have not been identified. Here, we show that metabolic features determine sensitivity to the PI3K/mTOR dual inhibitor gedatolisib in SCLC cells. Substantial phosphatidyl lipid analysis revealed that a specific phosphatidylinositol (3,4,5)-trisphosphate (PIP3) sub-species lipid product, PIP3(38:4) is predictive in assessing sensitivity to PI3K/mTOR dual inhibitor. Notably, we found that higher amounts of purine-related aqueous metabolites such as hypoxanthine, which are characteristic of SCLC biology, lead to resistance to PI3K pathway inhibition. In addition, the levels of the mRNA encoding hypoxanthine phosphoribosyl transferase 1, a key component of the purine salvage pathway, differed significantly between SCLC cells sensitive or resistant to gedatolisib. Moreover, complementation with purine metabolites could reverse the vulnerability to targeting of the PI3K pathway in SCLC cells normally sensitive to gedatolisib. These results indicate that the resistance mechanism of PI3K pathway inhibitors is mediated by the activation of the purine salvage pathway, supplying purine resource to nucleotide biosynthesis. Metabolomics is a powerful approach for finding novel therapeutic biomarkers in SCLC treatment.

Significance: These findings identify features that determine sensitivity of SCLC to PI3K pathway inhibition and support metabolomics as a tool for finding novel therapeutic biomarkers.

Introduction

Small-cell lung cancer (SCLC) accounts for approximately 14% of all lung cancers. It is an exceptionally aggressive neuroendocrine tumor with a high proliferative index and a strong prediction for early metastasis (1, 2). Basic and clinical research have produced little innovation in the treatment of SCLC over the past 30 years (3–5). Recent studies reporting a comprehensive genomic analysis of SCLC suggested that transcriptional deregulation in the PI3K/AKT/mTOR signaling pathway plays a key role in cell proliferation, growth, survival, and protein synthesis (10–13). It not only provides strong growth and survival signals to tumor cells but also has profound effects on glucose and amino acid metabolism (13–17). Class I PI3Ks play a key role in the biology of human cancer. They are responsible for the production of phosphatidylinositol 3-monophosphate [PI(3)P], phosphatidylinositol 3,4-bisphosphate [PI(3,4)P2], and phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3]; refs. 12, 18. The gene encoding the PI3K-α isoform (PIK3CA) is mutated or amplified in a wide range of cancers (10, 12). Because the PI3K/AKT/mTOR pathway is distinguishable among SCLC genomic alterations (19–21), it may be a feasible chemotherapeutic target. An investigator-initiated phase II study of gedatolisib (PF-05212384, PKI-587) in advanced recurrent SCLC patients harboring activating mutations in this pathway is ongoing (University Hospital Medical Information Network trial number 000020585). Gedatolisib is a highly potent dual inhibitor of PI3K and mTOR, and it has shown excellent activity in vitro and in vivo, with antitumor efficacy in both subcutaneous and orthotopic xenograft tumor models when administered intravenously (22). Its efficacy, pharmacokinetic, and safety profiles are favorable, and it has advanced to phase I clinical evaluation (23), and the agent afforded acceptable tolerability and activity in patients with recurrent endometrial cancer in a phase II study (24).
Metabolomics, which can be defined as measurement of the levels of all cellular metabolites, is a comprehensive assessment of endogenous metabolites. It attempts to systematically identify metabolites from clinical samples (25–27). Dysregulated metabolism is a hallmark of cancer, with numerous metabolic alterations found in tumor cells with respect to aerobic glycolysis, reduced oxidative phosphorylation, and increased generation of the bio-synthetic intermediates needed for cell growth and proliferation (28–34). Metabolomics has been applied to cancer diagnosis and drug discovery with the goal of identifying pathways linked to cancer progression. We recently reported that treatment with kinase inhibitors altered levels of numerous metabolites in drug-sensitive, but not drug-resistant, lung adenocarcinoma cells (15, 35).

The development of new and more potent PI3K/AKT/mTOR-targeted therapies for SCLC requires the availability of predictive biomarkers able to select the most effective therapies for different patients. Here, we show that the PI3K/mTOR dual inhibitor gedatolisib effectively represses SCLC tumor growth in vivo and that metabolic biomarker levels can predict the response to this PI3K/mTOR inhibitor.

Materials and Methods

Patient samples

This study has been performed in compliance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards, and Japanese ethical guidelines for human genome/gene analysis research issued by Ministry of Education, Culture, Sports, Science and Technology, and Ministry of Health, Labour and Welfare of Japan. All clinical specimens were collected from patients after obtaining written informed consent for the use of their biological material. The National Cancer Center Institutional Review Board approved this study. The Institutional Review Board approval number of this study is 2011-201.

Metabolite measurements

Tumor tissues and surrounding nontumor tissues were surgically resected or acquired from biopsies of SCLC patients. The tumor specimens comprised >40% tumor cells. Unpaired nontumor lung tissues were pathologically proven nonmalignant tissues obtained from pulmonary nodules or tumors. We used adenocarcinoma cell lines to compare the metabolic profiles of SCLCs to those of adenocarcinomas (35). Xenograft SCLC tumors were resected from mice. The resected tumor samples were immediately frozen in liquid nitrogen, weighed, and stored at –80°C until metabolite extraction. Metabolic extracts were prepared from 1 to 10 × 10^6 SCLC cells with methanol containing Internal Standard Solution (Human Metabolome Technologies) and analyzed using a capillary electrophoresis (CE)–connected electrospray ionization/time-of-flight mass spectrometry and capillary electrophoresis tandem mass spectroscopy (CE-MS/MS) system (CARCINOSCOPE, Human Metabolic Technologies). Procedures for metabolite measurements were as previously described (15, 35) and are described in more detail in the Supplementary Information.

Phosphoinositide measurements

Cells were placed in lysis buffer on ice and sonicated. The protein content of supernatants was quantified, and a solution containing 3 mg protein was used for further experiments.

Phosphatidylserine (PS), phosphatidylinositol (PI), PI monophosphate (PIP), PI bisphosphate (PIP2), and PI trisphosphate (PIP3), containing 3 mg protein was used for further experiments.

Statistical analysis

We used the Student t test, Welch t test, and two-sided Mann–Whitney U tests. Mann–Whitney U tests were conducted by SPSS version 22. A P value < 0.05 was considered statistically significant unless stated otherwise. We used Ward’s hierarchical clustering method to classify gedatolisib-sensitive and -resistant cell lines.

Metabolite set enrichment analysis

Metabolite set enrichment analysis (MSEA) was performed according to a published protocol (38). Briefly, the targeted metabolites were classified into 16 categories according to their metabolic pathways (e.g., glycolysis) or metabolite groups (e.g., essential amino acids). MSEA was performed using R (v2.15.3) with the msea package (https://cran.r-project.org/web/packages/msea). The classification of metabolic pathways is listed in Supplementary Table S1.

Results

Genomic alterations and sensitivity to gedatolisib in SCLC cell lines

To investigate the efficacy of a PI3K/AKT/mTOR pathway inhibitor against SCLC, we investigated whether alterations in this pathway enhanced the sensitivity of SCLC cell lines to the PI3K/mTOR dual inhibitor gedatolisib. We analyzed the in vitro sensitivity of SCLC cell lines to the dual PI3K/mTOR kinase inhibitor gedatolisib through proliferation assays. The genomic profiles of the SCLC cell lines used in this
article are shown (Supplementary Table S2). IC_{50} values were measured in 42 SCLC cell lines and found to range from 5 nmol/L to $\geq 1$ $\mu$mol/L (Fig. 1A). We previously reported that NCI-H1048 cells harboring an activating mutation in the PIK3CA gene (H1047R) were the most sensitive to BEZ235 (PI3K/mTOR dual inhibitor), with an IC_{50} of 5.4 nmol/L (20). Consistent with this result, we confirmed that H1048 was the SCLC cell line most sensitive to gedatolisib treatment. We found that SCLC cell lines could be categorized into two groups according to their susceptibility to gedatolisib treatment: four of the cell lines (H1048, DMS114, H1187, and H446) were sensitive (mean IC_{50} = 14.9 nmol/L), with an IC_{50} value after 72 hours of treatment of $\leq 23.6$ nmol/L, whereas the other 38 SCLC cell lines were resistant to gedatolisib treatment (mean IC_{50} = 219.5 nmol/L), with an IC_{50} value after 72 hours of treatment of $\geq 45.8$ nmol/L (Fig. 1A). Half of the gedatolisib-sensitive cell lines (H1048 and H446) harbored genomic alterations in the signaling molecules of the PI3K pathway (PIK3CA H1047R mutation and PTEN deletion), as did 14 gedatolisib-resistant SCLC cell lines (e.g., SBC-5 and H1694; Fig. 1A). Two cell lines, DMS53, which harbors the H1047R mutation and PIK3CA (H1047R), were the most sensitive to BEZ235 (PI3K/mTOR dual inhibitor), with an IC_{50} of 5.4 nmol/L (20). Consistent with this result, we confirmed that H1048 was the SCLC cell line most sensitive to gedatolisib treatment. We found that SCLC cell lines could be categorized into two groups according to their susceptibility to gedatolisib treatment: four of the cell lines (H1048, DMS114, H1187, and H446) were sensitive (mean IC_{50} = 14.9 nmol/L), with an IC_{50} value after 72 hours of treatment of $\leq 23.6$ nmol/L, whereas the other 38 SCLC cell lines were resistant to gedatolisib treatment (mean IC_{50} = 219.5 nmol/L), with an IC_{50} value after 72 hours of treatment of $\geq 45.8$ nmol/L (Fig. 1A). Half of the gedatolisib-sensitive cell lines (H1048 and H446) harbored genomic alterations in the signaling molecules of the PI3K pathway (PIK3CA H1047R mutation and PTEN deletion), as did 14 gedatolisib-resistant SCLC cell lines (e.g., SBC-5 and H1694; Fig. 1A). Two cell lines, DMS53, which harbors the KRAS amplification, and SHP77, which harbors the KRAS G12V mutation, were extremely resistant to gedatolisib, suggesting that activation of the KRAS pathway might induce a gedatolisib-resistant phenotype in SCLC cell lines. No correlation was observed between gedatolisib sensitivity and copy-number gain in the MYC family gene, which is widely distributed in SCLC cell lines (Fig. 1A). Likewise, there was no apparent relationship between gedatolisib sensitivity and mutation or amplification/deletion of the major cancer genes, such as RB1 or TP53, among the SCLC cell lines (Supplementary Table S2). In addition, there was no correlation between gedatolisib sensitivity and sensitivity to the cytotoxic agent etoposide (Fig. 1B).

### The phosphorylation status of proteins and sensitivity to gedatolisib in SCLC cell lines

To define the connectivity of protein phosphorylation networks, we quantitatively delineated the PI3K and mTOR network in SCLC cell lines by mass spectrometry–based phosphoproteomics. We quantitated levels of phospho-peptides using this proteomic approach after H1048 cells were treated with DMSO vehicle or gedatolisib (100 nmol/L) for 1 hour in triplicate. A total of 4,185 phosphorylated peptides were identified in H1048 cells under these experimental conditions (Supplementary Table S3). The first threshold was set at the statistical significant level for the $P$ value using Student t tests ($P < 0.05$). The phosphorylation level of proteins downstream of mTOR such as 4EBP1, 4EBP2, ULK1,
RPS6, and CAD was significantly decreased after treatment with gedatolisib as compared with DMSO, when the cutoff point was set to 1.7-fold (Fig. 2A). Moreover, the phosphorylation of AKT target proteins, which have the phospho-AKT substrate motif (RXXS/I), was also downregulated in H1048 cells treated with gedatolisib (Fig. 2A). Thus, gedatolisib treatment inhibited both AKT and mTOR kinase activities in H1048 cells under these experimental conditions.

To validate the phosphoproteomic data, we used Western blot analysis to again assess for gedatolisib inhibition of AKT activity, mTOR activity, or related signaling molecules by using four gedatolisib-sensitive and five gedatolisib-resistant cell lines. Amounts of total AKT, phospho (p)-AKT (T308), p-AKT (S473), total 4EBP1, p-4EBP1, and GAPDH were determined in cells treated with DMSO or gedatolisib (100 nmol/L) for 2 hours. We noticed that phosphorylation of AKT at S473 by mTORC2 was detected in all SCLC cell lines under normal growth conditions, but that p-T308 AKT, which is phosphorylated by PDK1, was not detected in the two resistant cell lines, DMS53 and SHP77 (Fig. 2B). Despite equivalent amounts of total AKT, p-T308, and p-S473 AKT were clearly suppressed by gedatolisib in the four sensitive cell lines and even in three of the five resistant cell lines tested (H69, H1092, and H1694 cells; Fig. 2B). Phosphorylation of the downstream signaling molecule 4EBP1 was also inhibited by the addition of gedatolisib to H1048, DMS114, H187, H446, H69, H1092, and H1694 cells but not to the extremely resistant DMS53 and SHP77 cells (Fig. 2B). Thus, treatment with gedatolisib inactivated the PI3K pathway in all four sensitive cell lines, but also in three resistant cell lines. Taking these findings together, we concluded that protein phosphorylation status was not concordant with sensitivity to gedatolisib in SCLC cells.

Figure 2.
Gedatolisib treatment changes phosphorylation status in the proteins involved in the PI3K pathway. A, Quantitative phosphoproteomics was carried out, and relative abundance of representative phospho-peptides is shown (threshold, 1.7-fold). Student t tests were used to calculate which phospho-peptides were significantly decreased or increased in H1048 cells (n = 3) treated with gedatolisib (100 nmol/L, 2 hours), relative to DMSO control (P < 0.05). B, Phosphorylation status involving PI3K pathway proteins in gedatolisib-sensitive and gedatolisib-resistant SCLC cell lines treated with or without gedatolisib. SCLC cells were treated with DMSO or gedatolisib (100 nmol/L) for 2 hours, and whole-cell lysates were analyzed by Western blotting with antibodies to described proteins. GAPDH served as a loading control.
Comprehensive phosphatidyl lipid analysis revealed that a specific PIP2 subspecies was a definitive lipid product to assess sensitivity to gedatolisib. Class I PI3Ks function by phosphorylating the inositol ring of PIP2 to generate the second messenger PIP3, which is dephosphorylated by the phosphatase PTEN, generating PIP2.

The discrepancy between in vitro and in vivo efficacy of gedatolisib against SCLC cells is consistent with its efficacy in vitro. The mean baseline PIP3 (38:4) level was higher in H1048 tumors [0.227 pmol/mg (tissue)] than in SBC-5 tumors [0.038 pmol/mg (tissue); Supplementary Fig. S1]. The mechanism of growth inhibition of SCLC tumor growth by gedatolisib was further examined by IHC. Representative IHC images of gedatolisib-treated tumors revealed a dramatic change in SCLC pathology in the H1048-derived tumors at 6 hours after gedatolisib treatment (Fig. 4C). Intravenous injection of gedatolisib to H1048-xenografted mice induced significant apoptosis, as revealed by cleaved PARP (cPARP) in the H1048-derived tumors, that was not observed in control mice administered the vehicle. In contrast, cPARP was not increased in resistant SBC-5 tumors (Fig. 4C), indicating that gedatolisib suppresses H1048 tumor growth through apoptotic mechanisms. Taken together, these results prove that the PI3K pathway has an important role for SCLC tumor growth and cell survival in vivo.

Metabolic changes in an in vitro or in vivo model by gedatolisib treatment

We next aimed to assess which metabolic pathways were altered after inhibition of PI3K in both sensitive and resistant SCLC cells, by measuring the levels of 116 metabolites in the presence or absence of gedatolisib across three independent experiments. The ratios of metabolite levels observed for gedatolisib treatment and DMSO control are shown in logarithmic scale (Supplementary Fig. S2A). Between 24 and 41 metabolites (P < 0.05) were significantly altered with or without treatment with gedatolisib (Supplementary Table S6). Three metabolites phosphoribosyl diphosphate (PRPP), ADP-Ribose (ADP-Rib), and lactate were decreased in the gedatolisib-sensitive cell lines but not in the gedatolisib-resistant cell lines (Supplementary Fig. S2A). Despite equivalent levels of ATP, the levels of AMP, carbamoyl-aspartate (CM-Asp), and putrescine were decreased in both sensitive and resistant SCLC cell lines (Supplementary Fig. S2A). Moreover, various amino acids were increased in both sensitive and resistant SCLC cell lines after treatment with gedatolisib (Supplementary Fig. S2A).

To confirm the metabolic alterations observed in the in vitro experiments (Supplementary Fig. S2A), the metabolic profile was analyzed in tumors derived from sensitive or resistant SCLC cells transplanted into nude mice with or without gedatolisib treatment (Supplementary Table S7). The absolute amount of 116 extracted metabolites was obtained in three to five independent experiments (H1048 cells: n = 5, SBC-5 cells: n = 3). In H1048 xenografts sensitive to gedatolisib, 35 metabolites exhibited statistically significant changes after treatment with gedatolisib (P < 0.05, Supplementary Table S7). Although in SBC-5 xenografts resistant to gedatolisib, 9 of 116 metabolites were altered with or without treatment (P < 0.05, Supplementary Table S7).

Putrescine was decreased in both sensitive and resistant SCLC xenografts (Supplementary Fig. S2B). Several amino acids accumulated in both sensitive and resistant SCLC xenografts (Supplementary Fig. S2B), consistent with the in vitro experiments (Supplementary Fig. S2A). Taken together, the reduction in PRPP...
levels and the accumulation of amino acids appear to be a characteristic metabolic signature of SCLC after treatment with a PI3K/mTOR dual inhibitor.

Metabolic determinants of sensitivity to PI3K pathway inhibitor in SCLC

We generated a large-scale metabolomic data set for 28 SCLC cell lines, together with the pharmacologic IC50 of gedatolisib across 42 SCLC cell lines. To evaluate differences in characteristic metabolites in SCLC cells and lung adenocarcinoma cells, we compared metabolic profiles between 4 lung adenocarcinoma cell lines (35) and 28 SCLC cell lines, including resistant SCLC cell lines, and classified them using partial least squares discriminant analysis (Supplementary Fig. S3). As shown in Supplementary Fig. S3, endogenous metabolites in the lung adenocarcinoma cells clearly differed from those in the SCLC cells, indicating that SCLC cells have a metabolic profile distinct from lung adenocarcinoma cells. The trajectory of the sensitive SCLC cells was distinguishable from the position of the resistant SCLC cells according to latent variable 2 (LV2; Supplementary Fig. S3), suggesting that the
metabolic profile in sensitive SCLC cells is unique when compared with resistant SCLC cells.

To identify a metabolic biomarker for the prediction of gedatolisib sensitivity, we compared levels of various metabolites between sensitive and resistant SCLC cells before gedatolisib treatment. Under normal growth conditions, the levels of metabolites in central carbon metabolism were higher in the sensitive SCLC cell lines than in the resistant SCLC cell lines (Fig. 5A). On the other hand, purine-related metabolites such as HXT, AMP, and GMP were significantly lower (P < 0.05) in sensitive than in resistant SCLC cell lines (Fig. 5A). Another purine-related metabolites, adenyl-succinate (Ad-SA), XMP, IMP, guanine (GUA), and NADP+, were marginally significantly lower (P < 0.1) in sensitive than in resistant SCLC cell lines (Fig. 5A). Although the amount of UA was high in sensitive SCLC cells, its precursor, HXT, was present at low levels in these cells, although HXT levels were relatively higher in the resistant SCLC cells (Fig. 5A). To identify significantly enriched metabolic pathways related to gedatolisib sensitivity in SCLC cells, we performed MSEA. Purine metabolism was significantly altered in sensitive compared with resistant SCLC cell lines (P < 0.001, q value < 0.01; Fig. 5A; Supplementary Table S8).

To reveal which metabolic pathway was important in determining sensitivity to gedatolisib, we analyzed gene expression with or without gedatolisib treatment in sensitive H1048 cells and resistant H1694 cells. RNA sequence analysis showed that the expression level of genes involved in the Forkhead transcription factor (FOXO) pathway was increased at 3 hours after treatment with gedatolisib in H1048 cells as compared with H1694 cells (Supplementary Fig. S4A), consistent with previous findings (41). Under this experimental condition, genes related to the methionine cycle and the polyamine biosynthesis pathway were downregulated in H1048 cells after treatment with gedatolisib (Supplementary Fig. S4A). MAT and AMD1 are involved in the methionine cycle, and SRM has an important role in spermidine and methylthioadenosine (MTA) biosynthesis in the purine salvage pathway (Supplementary Fig. S5). To confirm the RNA sequence data, we measured AMD1, SRM, and MAT2A mRNA level using the reverse-transcription PCR. The relative levels of AMD1, SRM, and MAT2A mRNA level using the reverse-transcription PCR. The relative levels of AMD1, SRM, and MAT2A mRNA were significantly downregulated in gedatolisib-treated H1048 cells, but were not changed in resistant H1694 cells (Supplementary Fig. S4B). In addition, the expression of AMD1 was significantly decreased in the sensitive DMS114 cells after treatment (Supplementary Fig. S4B). Taken together, these findings showed that the expression of enzymes involved in polyamine biosynthesis and the methionine cycle was decreased in gedatolisib-sensitive SCLC cells after inhibition of the PI3K pathway.
The purine salvage pathway is linked with the methionine cycle and polyamine biosynthesis pathways (Supplementary Fig. S5). Gedatolisib treatment significantly decreased the levels of PRPP in gedatolisib-sensitive but not gedatolisib-resistant cell lines (Supplementary Fig. S2A and S2B). Those results led us to hypothesize that the addition of MTA changed the gedatolisib sensitivity of SCLC cells. To test this, we added MTA to the culture medium and measured survival of the H1048- and DMS114-sensitive cell lines after treatment with 10 or 100 nmol/L of gedatolisib. Although purine metabolites were supplied through either the de novo or salvage pathway, we also considered the possibility that the purine salvage pathway might be downregulated during purine biosynthesis after gedatolisib treatment. As predicted, the addition of MTA to the culture media of two drug-sensitive cell lines, H1048 and DMS114, resulted in a more gedatolisib-resistant phenotype, as reflected by increased cell survival (Fig. 5C). In contrast, the addition of MTA to the culture media of the drug-sensitive cell lines DMS273 and SBC-5 resulted in a more gedatolisib-sensitive phenotype (Supplementary Fig. S6A and S6B). These results indicate that the vulnerability to targeting of the PI3K pathway in SCLC cells could be purine biosynthesis pathway.

Figure 5.
A, Relative abundance of key metabolites in gedatolisib-sensitive SCLC cells, normalized to gedatolisib-resistant SCLC cells. Mann–Whitney U tests were used to calculate which metabolites were significantly lower or higher in gedatolisib-sensitive SCLC cells, relative to gedatolisib-resistant SCLC cells (P < 0.05). Red dots, purine-related metabolites; green dots, amino acids; and blue dots, intermediate metabolites in the central carbon metabolism. B, Pie chart representing the results of MSEA to identify significantly enriched metabolic pathways related to the sensitivity of SCLC cells to gedatolisib. The levels of metabolites that were significantly lower (0.5-fold) or higher (2-fold) in gedatolisib-sensitive compared with those of resistant SCLC cells were selected. The number of metabolites selected in each metabolic pathway is shown. The Fisher exact test was used to evaluate the statistical significance of the enrichment of a pathway (P < 0.05). C, Addition of MTA changed drug sensitivity from a sensitive to resistant phenotype. H1048 or DMS114 cell survival (%) was measured in the presence of 0.1 mmol/L MTA cotreated with either 100 or 10 nmol/L of gedatolisib. The data are shown as the mean ± SD (n = 6). Error bars, one SD. **, P < 0.01; ***, P < 0.001, t test.
Together, these results suggest a potential molecular mechanism through which gedatolisib treatment downregulates the de novo purine biosynthesis pathway. Even though gedatolisib inhibits the de novo purine biosynthetic pathway, the purine salvage pathway also supplies purine resources required for the biosynthesis of purine nucleotides (Fig. 6A). To verify this idea, we focused on the purine salvage pathway, because the characteristic metabolic feature of gedatolisib-resistant SCLC cells was the high levels of hypoxanthine (Fig. 6A). Notably, the levels of the mRNA encoding hypoxanthine phosphoribosyl transferase 1 (HPRT1), a key component of the purine salvage pathway, differed significantly between cells sensitive or resistant to gedatolisib ($P = 0.00311$; Fig. 6B). We thought that the high level of HPRT1 expression might cause drug resistance of SCLC cells. To test this idea, we measured the cellular sensitivity to gedatolisib after introduction of siRNAs including nontargeting control (NC) or three sets of HPRT1 sequences. siRNA targeting HPRT1 successfully knocked down HPRT1 mRNA and HPRT1 in DMS273 cells compared with the negative control expressing siNC (Supplementary Fig. S7A and S7B). Surprisingly, loss of HPRT1 in SCLC cells decreased cellular proliferation in the presence of gedatolisib compared with the nontargeting control (Fig. 6C). Further, the level of HPRT1 was higher in the gedatolisib-resistant tumors than the sensitive tumors (Supplementary Fig. S7C). These results indicate that the resistance mechanism of PI3K pathway inhibitors is mediated by the activation of the purine salvage pathway, supplying purine resource to nucleotide biosynthesis independent of de novo purine biosynthesis (Fig. 6A).

### Metabolic profiles of clinical samples of SCLC tissues

To evaluate SCLC-specific metabolism, we compared the levels of 116 hydrophilic metabolites using liquid chromatography coupled with tandem mass spectrometry methods from training four matching pairs of high-quality surgically resected fresh and frozen SCLC tumors and adjacent nontumor tissues (same number as training set). We further analyzed the levels of these 116 metabolites in an independent test set of 17 samples that were obtained from 13 SCLC tumors (2 surgically resected and 11 taken from biopsies of patients with advanced disease) and unpaired 4 nontumor tissues (same number as the testing set; Supplementary Table S9). We initially performed principal component analysis of the 116 metabolites obtained from each sample to discern the presence of inherent similarities in SCLC metabolic profiles. Five metabolites were not detected in any cases (Supplementary Table S9). Statistically significant differences between SCLC tumor tissues and adjacent nontumor tissues were observed in 46 metabolites, and the relative levels of these metabolites in tumor versus nontumor tissue were plotted (Fig. 7). It was characteristic that purine-related metabolites, such as hypoxanthine, were higher in SCLC tumor tissues. Purine-related metabolites are characteristic of SCLC biology, which might serve as novel therapeutic biomarkers such as the PI3K pathway.

### Discussion

Our studies shown in this article revealed that higher levels of purine pathway–related metabolites correlate with SCLC sensitivity to a PI3K pathway inhibitor. This shows that sensitivity to gedatolisib is determined by cellular metabolic characteristics, including both hydrophilic metabolites. We also showed that complementation with purine metabolites changes the sensitivity to gedatolisib from sensitive SCLC cells to resistant phenotype. The purine salvage pathway, which might be activated in gedatolisib-resistant cells, was associated with resistance to the PI3K pathway inhibitor, which was indicated by the high levels of HPRT1 mRNA in resistant cells. Finally, our results indicated that the level of purine-related metabolites can be potential biomarkers for a response to inhibitors targeting the PI3K/AKT/mTOR pathway.

We demonstrated that treatment with gedatolisib effectively repressed the growth of H1048 cells harboring active PIK3CA mutations such as H1047R in the kinase domain. Gedatolisib inhibits PI3K, mTOR, and its downstream signaling molecules in SCLC cells (Fig. 2B) and induces apoptosis in H1048 tumor xenografts (Fig. 4C). It is still unclear how the genomic profiles affect signaling through the PI3K pathway. Indeed, half of the gedatolisib-sensitive cell lines did not harbor genomic alterations in the PIK3/ AKT/mTOR pathway (Fig. 1A). Because this population cannot be detected by genomic screening alone, the provision of targeted therapies to SCLC patients requires more feasible biomarkers of sensitive populations without omission. Metabolomics is considered to be more representative to the actual phenotype compared with proteomics or genomics data (42). Although statin, a microtubule-regulatory protein associated with HIF-1 alpha levels, was used as a biomarker for gedatolisib in a phase II study of patients with recurrent endometrial cancer, high statin expression did not correlate with greater treatment efficacy (24). A proteomic approach to explore biomarkers of PI3K inhibitors was unsuccessful in this study; however, changes in glucose homeostasis consistent with a PI3K blockade were observed following administration of gedatolisib, suggesting the usefulness of metabolic biomarkers. For reasons such as these, a metabolomic approach for discovering novel therapeutic biomarkers of this pathway is feasible.

The activated PI3K and mTOR pathway induces vigorous cellular metabolism to build up macromolecules in response to growth signals in a variety of cancers (14, 17, 31). We have previously shown that mutant EGFR signaling in lung adenocarcinoma cells maintained upregulated aerobic glycolysis, PPP, and de novo pyrimidine biosynthesis through the PI3K pathway (15, 35). Here, we show that sensitivity to gedatolisib is determined by the global metabolic profile, including both hydrophobic and hydrophilic metabolite composition. Specifically, the level of PIP3_38:4 was higher between gedatolisib-sensitive and gedatolisib-resistant SCLC (Fig. 3C). It is reported that the elevated level of PIP3 is related to PI3K/AKT/mTOR pathway activation (36). Our results suggest that PIP3_38:4 is crucial to activation of the PI3K pathway, suggesting its utility as an indicator for predicting sensitivity to PI3K pathway inhibitors such as gedatolisib. PIP3_38:4 levels were evaluated in xenograft tumors. Direct measurement of PIP3_38:4 in a recent study revealed an unexpected role of PI3K in luminal breast cancer (39). Although the ELISA method does not allow for the determination of molecular species, we could identify it by analyzing the PIP profile using ESI mass spectrometry. ESI mass spectrometry might become a more specific and promising method for future biomarker analysis.

We showed that high levels of purine-related metabolites allowed SCLC cells to become resistant to the PI3K pathway inhibitor. Purines, including adenine nucleotides, guanine nucleotides, cAMP, NAD, HXT, and xanthine, are a class of small organic molecules that are essential for all cells. These molecules play central roles as building blocks for DNA and RNA during cell proliferation, serve as cofactors for many enzymatic reactions, and act as both intracellular and intercellular signaling messengers (43). Purine
metabolism is linked to the PI3K pathway, which regulates the early steps of de novo synthesis by modulating PRPP production and the activity of enzymes such as IMP cyclohydrolase and transketolase (44, 45). It has been shown that mTORC1 induces de novo purine biosynthesis through the transcriptional factor ATF4 (46). The methionine cycle and polyamine biosynthesis pathway links to the salvage pathway through MTA biosynthesis (47, 48). Gedatolisib reduced putrescine and PRPP in vitro and in vivo in sensitive H1048 cells (Supplementary Fig. S2A and S2B). This is likely due to the decreased purine salvage pathway in gedatolisib-sensitive cells. In that case, drug sensitivity to gedatolisib would be altered if an exogenous purine precursor compound were added. Indeed, adding MTA induced a more resistant phenotype in the gedatolisib-sensitive SCLC cells (Fig. 5C). It is possible that gedatolisib treatment reduced putrescine and PRPP in vitro and in vivo in sensitive H1048 cells (Supplementary Fig. S2A and S2B). This is likely due to the decreased purine salvage pathway in gedatolisib-sensitive cells. In that case, drug sensitivity to gedatolisib would be altered if an exogenous purine precursor compound were added. Indeed, adding MTA induced a more resistant phenotype in the gedatolisib-sensitive SCLC cells (Fig. 5C). It is possible that gedatolisib treatment

Figure 6. 
A, Diagram depicting a potential mechanism by which the treatment of gedatolisib downregulates de novo purine biosynthetic pathway by inhibiting the PI3K pathway, and the purine salvage pathway also supplies purine resources required to biosynthesize purine nucleotides. 
B, Levels of HPRT1 mRNAs in gedatolisib-sensitive (red bars) and -resistant (blue bars) cells differed significantly. The data are shown as the mean ± SD (n = 3). Error bars, one SD. P = 0.00311, t test. 
C, Involvement of HPRT1 in gedatolisib sensitivity. DMS273 cells transfected with HPRT1 siRNAs were incubated for 2 days, and cell survival was measured in the presence of gedatolisib, 100 nmol/L (left) or 10 nmol/L (right), 72 hours after treatment. The data are shown as the mean ± SD (n = 4). *, P < 0.05; **, P < 0.01 (Student t test).
SCLC is characterized as a tumor with cells that have a small size, a round-to-fusiform shape, scant cytoplasm, and finely granular nuclear chromatin. In addition, mitotic rates are high, with an average of 80 mitoses per 2-mm² area (52). This means that nucleic acid synthesis is especially active in SCLC. Purine metabolism, which plays a pivotal role in nucleic acid synthesis, might be the metabolic pathway that captures the characteristic feature of SCLC biology (Fig. 7). Accordingly, measurement of the level of purine-related metabolites is important not only to identify potential biomarkers of PI3K/mTOR pathway inhibitors but also for exploring other novel therapeutic strategies. Further research to investigate the molecular mechanisms that regulate purine metabolism in SCLC cells is warranted. Our findings indicate that the purine-related metabolic pathway is a vulnerability of SCLC, thus this pathway could be promising for future therapeutic targets.

Disclosure of Potential Conflicts of Interest
T. Sasaki is CEO at ALTe, LLC. K. Goto has honoraria from the speakers bureau of Pfizer. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Makinoshima, S. Umemura, H. Nakanihshi, A. Manyama, S. Mimaki, M. Tsuibo
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Acknowledgments
Gedatolisib was obtained with a material transfer agreement from Pfizer. We wish to thank Drs. Shogo Nomura, Kaisuke Kiriti, Kiyotaka Yoh, Phil Wong, and Hitobu Ohmatsu for their support and comments. We wish to thank HMT, ALTe, and TechnoPro for professional assistance. This study was performed as a research program of the Project for Development of Innovative Research on Cancer Therapeutics (P-Direct). This study was supported by the Ministry of Education, Culture, Sports, Science and Technology, Japan, Ministry of Health, Labour and Welfare, Japan, Japan Agency for Medical Research and Development (AMED), the National Cancer Center Research and Development Fund (28–A–9), and by funds from Yamagata Prefecture and Tsuruoka City.

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Received July 17, 2017; revised December 15, 2017; accepted February 22, 2018; published first February 28, 2018.

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Cancer Res 2018;78:2179-2190. Published OnlineFirst February 28, 2018.

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