Inhibition of PI3K Pathway Reduces Invasiveness and Epithelial-to-Mesenchymal Transition in Squamous Lung Cancer Cell Lines Harboring PIK3CA Gene Alterations

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Abstract

A prominent role in the pathogenesis of squamous cell carcinoma of the lung (SQCLC) has been attributed to the aberrant activation of the PI3K signaling pathway, due to amplification or mutations of the p110α subunit of class I phosphatidylinositol-3-kinase (PIK3CA) gene. The aim of our study was to determine whether different genetic alterations of PIK3CA affect the biologic properties of SQCLC and to evaluate the response to specific targeting agents in vitro and in vivo. The effects of NVP-BEZ235, NVP-BKM120, and NVP-BYL719 on two-dimensional/three-dimensional (2D/3D) cellular growth, epithelial-to-mesenchymal transition, and invasiveness were evaluated in E545K or H1047R PIK3CA–mutated SQCLC cells and in newly generated clones carrying PIK3CA alterations, as well as in a xenograft model. PIK3CA mutated/amplified cells showed increased growth rate and enhanced migration and invasiveness, associated with an increased activity of RhoA family proteins and the acquisition of a mesenchymal phenotype. PI3K inhibitors reverted this aggressive phenotype by reducing metalloproteinase production, RhoA activity, and the expression of mesenchymal markers, with the specific PI3K inhibitors NVP-BKM120 and NVP-BYL719 being more effective than the dual PI3K/mTOR inhibitor NVP-BEZ235. A xenograft model of SQCLC confirmed that PIK3CA mutation promotes the acquisition of a mesenchymal phenotype in vivo and proved the efficacy of its specific targeting drug NVP-BYL719 in reducing the growth and the expression of mesenchymal markers in xenotransplanted tumors. These data indicate that PIK3CA mutation/amplification may represent a good predictive feature for the clinical application of specific PI3K inhibitors in SQCLC patients. Mol Cancer Ther; 14(8); 1–12. ©2015 AACR.

Introduction

Squamous cell carcinoma of the lung (SQCLC) accounts for approximately 30% of non–small-cell lung cancer (NSCLC) and it is almost invariably associated with smoking (1). Currently, there are no effective molecular-targeted therapies for this subset of patients and platinum-based chemotherapy remains the standard of care treatment for advanced disease. These observations point out the urgency for the identification of targetable pathways relevant for the pathogenesis of SQCLC, whose inhibition could be exploited as a new and suitable therapeutic strategy. A new option for patients with SQCLC is represented by the blockade of immune regulatory checkpoints such as PD-1. Very recently for SQCLC patients, whose disease had progressed during or after treatment with platinum-based chemotherapy, the FDA approved the anti–PD-1 antibody Nivolumab, that increased median overall survival up to 9.2 months, compared with 6 months for chemotherapy (2).

Genomic characterization studies displayed that several genetic alterations are frequently found in SQCLC (3) and aberrant activation of the PI3K signaling pathway shows a prominent role in the pathogenesis of SQCLC. Class I PI3Ks consists of a regulatory (p85) and a catalytic subunit (p110). The regulatory subunit binds to phosphotyrosine residues in the cytoplasmic domain of tyrosine kinase receptors (RTK) through its Src-homology 2 domains, which leads to PI3K activation. PI3Ks phosphorylate inositol lipids, generating phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3) that triggers signal transduction cascades by activating different protein kinases (4). Including AKT. By inhibiting the TSC1–TSC2 complex, AKT activates the serine-threonine kinase mTOR, that exists in two distinct complexes, mTORC1 and mTORC2, upon binding with different regulatory proteins (5).
Interestingly, both mTORC1 and mTORC2 complexes as well as PI3K are involved in the rearrangement of the cytoskeleton, and in the control of tumor cell migration and invasion (6).

Deregulation of the PI3K–AKT–mTOR pathway is more common in SQCLC than in other NSCLC histotypes and occurs through a variety of mechanisms, including activation of RTKs upstream of PI3K, PIK3CA amplification/mutation, loss of negative regulation by PTEN as well as mutations in AKT (7).

The most frequent PIK3CA alteration is gene amplification, found in about 30% of SQCLC (7–10). Mutations in PIK3CA are less common (3%–10% of SQCLC) and oncogenic alterations are frequently localized in exons 9 (E542K and E545K) and exon 20 (H1047R), which correspond to the helical domain and the kinase domain of p110α subunit, respectively (7, 9, 11–15). PTEN loss and PTEN inactivating mutations are reported in 8% to 59% and 3% to 10% of SQCLC, respectively (7, 9, 10, 14–17). AKT activating mutation is less frequent (1%–2%), whereas its overexpression involves 19% to 32% of SQCLC (6, 9).

On the basis of the critical role of the PI3K–AKT–mTOR axis in the control of cell growth, metabolism, and migration, the components of this pathway represent attractive candidates for targeted cancer agents. Currently, a number of PI3K inhibitors with different activity on PI3K isoforms and mTOR are emerging. Among these, NVP-BEZ235, classified as a dual PI3K/mTOR inhibitor, is a potent, reversible inhibitor of both class I PI3K and mTOR kinase catalytic activity by competing with the ATP-binding site (18). NVP-BKM120 is a potent class I PI3K pan-inhibitor and, unlike NVP-BEZ235, does not significantly inhibit mTOR (19). Finally, NVP-BYL719 is a specific inhibitor of the p110α subunit of class I PI3K (20).

The aim of the present study was to test whether inhibition of the PI3K–AKT–mTOR pathway exerts antitumor activity on SQCLC cells stably overexpressing wild-type or E545K- or H1047R-mutated forms of the PIK3CA gene in comparison with parental cells. Our results indicate that PI3K inhibitors reduced cell proliferation both in two-dimensional (2D) and three-dimensional (3D) systems independently from PIK3CA mutational status; in contrast, migrating and invading capabilities were reduced only in clones carrying alterations in PIK3CA gene. The acquisition of mesenchymal markers observed in clones with overexpressed or mutated PIK3CA gene was influenced by PI3K inhibitors that favored the maintenance of an epithelial phenotype both in vitro and in vivo. Our data suggest that motility and invasiveness of SQCLC cells are regulated by PI3K hyperactivation, which in turn prompts the EMT process. The inhibition of the PI3K pathway emerges as an important new therapeutic strategy to challenge the aggressive behavior of SQCLC.

Materials and Methods

Cell lines and drugs

Human SQCLC SKMES-1 and H596 cells were from the ATCC; the HCC2450 cell line was kindly provided by Dr. David Reisman (University of Florida, FL). Cells were typed by short tandem repeat profiling and never cultured for more than 3 months. Cells were maintained under standard culture conditions at 37°C in a water-saturated atmosphere of 5% CO₂ in air.

NVP-BEZ235, NVP-BKM120, and NVP-BYL719 (hereafter, referred to as BEZ235, BKM120 and BYL719) were provided by Novartis Institutes for BioMedical Research.

Plasmid transfection

SKMES-1 cells were transfected by standard electroporation procedure, as previously described (21). Cells were transfected with 10 μg of pCMV-Tag2A, pCMV2-PIK3CA-Tag2A, pCMV2-PIK3CA(E545K)-Tag2A, or pCMV2-PIK3CA(H1047R)-Tag2A plasmids (www.Addgene.org) and plated in growth medium with 600 μg/mL G418 (geneticin) antibiotic to generate stable clones expressing the PIK3CA genes.

Analysis of cell viability, cell proliferation, and cell death

Cell number, cell viability, and cell death were evaluated as previously described (22). Proliferation rates were estimated by the growth rate quotient (GRQ) as previously reported (23).

Western blotting analysis and pull-down assay

Procedures for protein extraction and protein analysis by 1-D PAGE are described elsewhere (24). For further antibodies information see Supplementary Materials and Methods. Active GTP-bound RhoA family proteins were obtained by using the Active Rho family Detection Kit from Cell Signaling Technology, accordingly to the manufacturer’s instructions.

The band intensity of the Western blotting was analyzed using QuantityOne software (Bio-Rad Laboratories Inc.).

Cell migration, invasion, and gelatine zymography

The migration and invasion assays and gelatine zymography were performed as previously described (23).

Spheroid generation and growth

Spheroids from SKMES-1 and from PIK3CA transfected clones were generated using LIPIDURE-COAT PLATE A-U96 (NOF Corporation) according to the manufacturer’s instruction. Briefly, 200 cells were seeded and after 1 to 3 days the spheroids were formed and treated with vehicle or drugs for 5 days. The effects of the drugs were evaluated in terms of volume changes using the Nikon Eclipse E400 Microscope with digital Net camera. The volume of the spheroids (V) was calculated as volume of spheres having as diameter the average of the maximum and minimum diameters D = (Dmax + Dmin)/2; V = 4/3π(D/2)³ obtained by measurement with ImageJ software (25).

Tumor spheroid-based migration assay

Tumor spheroid-based migration assay was performed as described by Vinci and colleagues (26). For detailed information see Supplementary Materials and Methods.

Tumor xenographs

A total of 5 × 10⁶ cancer cells were suspended in 200 μL of Matrigel (BD Biosciences) and PBS (1:1) and were s.c. injected in the right flank of Balb/c-Nude female mice (Charles River Laboratories). Ten mice were injected with SKMES-1 and 10 with clone 18; each animal developed a solid tumor at the inoculation site. One week after, when tumors reached an average size of 150 mm³, animals were randomized in control and treated groups (n = 5). BYL719 (30 mg/Kg in 0.5% methylcellulose), was administered daily by oрогastic gavage. Tumor xenographs were measured as previously described (27).

All experiments involving animals and their care were performed with the approval of the Local Ethical Committee of University of Parma, in accordance with the institutional
guidelines that are in compliance with national (DL116/92) and international (86/609/CEE) laws and policies.

Morphometric and immunoistochemical analysis of tumor xenografts

The volume fraction of intact and necrotic neoplastic tissue, fibrosis, and interstitial space was assessed on Masson’s Trichrome and hematoxylin and eosin (H&E)–stained samples. To this end, the number of points overlying each tissue component was counted and expressed as the percentage of the total number of points explored. Morphometric measurements were obtained with the aid of a grid defining a tissue area of 0.23 mm² and containing 42 sampling points each covering an area of 0.0052 mm².

Combining the entire tumor volume with the above morphometric measurements, the total volume occupied, respectively, by connective tissue, necrotic tissue, and vascular interstitium was computed on each sample.

Tumors were analyzed by double immunostaining to detect cells expressing cytokeratin7 (CK7) and vimentin (VIM). Sections were incubated with anti-VIM and anti-Ck7 antibodies, followed, respectively, by alkaline Phosphatase Red and DAB Detection Kits (Ventana, Roche). Finally, sections were counterstained with Mayer’s hematoxylin. A computer-assisted quantification was computed on micrographs of each section (Olympus-CK40; ×400 magnification). The area occupied by CK7pos and VIMpos cells in tumor sections was computed by a specific image analysis software (Image Pro-plus4.0, Media Cybernetics) detecting the overall brownish and the red signals corresponding to CK7 and VIM, respectively. A macroscopic image of the whole tumor section was captured to determine the total area of the nodule. Data are shown as fractional area occupied by cells expressing CK7 or VIM.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism 5.00 software. Statistical significance of differences among data was estimated by the two-tailed Student t test. Comparison among groups in the in vivo experiments was made using analysis of variance (two-way ANOVA, repeated measures) followed by the Bonferroni post-test; ***, P < 0.001; ***, P < 0.01; *, P < 0.05.

Results

Effects of BEZ235, BKM120, and BYL719 on proliferation and signaling transduction pathways in SQCLC cells harboring PIK3CA alterations

To evaluate the oncogenic potential of amplified wild-type (wt) or mutant forms of PIK3CA, we stably transfected SKMES-1 cells with plasmids containing wt, E545K or H1047R p110α subunit of PI3K or the empty vector. The activity of PI3K in amplified/mutated clones was evaluated in serum-starved cells; clones 1 and 10 carrying PIK3CA amplification, and clones 17, 18, and 28, 30 harboring the E545K and H1047R PIK3CA mutations, respectively, showed elevated levels of p-AKT(Ser473) compared with SKMES-1 cells transfected with the empty vector (Supplementary Fig. S1), indicating a constitutive activation of PI3K–AKT–mTOR signaling. The oncogenic potential of PI3K alterations was confirmed by the increased proliferation rates of PIK3CA transfected clones compared with SKMES-1 parental cells (Fig. 1A). Clones 10, 18, and 28 showed the highest increase in the GRQ and were selected for the subsequent experiments as representative models of PIK3CA oncogenic alterations. As shown in Fig. 1B, cells transfected with wt or mutant PIK3CA showed comparable increased levels of both p110α and p85α subunits of PI3K and increased p-p85 levels. As a consequence of PI3K activation, the levels of p-AKT both at Ser473 and Thr308 as well as of p-p70S6K were increased.

The effects of the PI3K inhibitors BKM120 and BYL719 and of the dual PI3K–mTOR inhibitor BEZ235 on cell proliferation were evaluated by MTT-assay. PI3K inhibitors impaired cell proliferation in a dose-dependent manner both in PIK3CA-transfected clones and in SKMES-1 cells. The concentration required to achieve 50% of the maximum cell proliferation arrest was of about 5 to 10 nmol/L for BEZ235 and 1 µmol/L for BKM120 and 1 to 2 µmol/L for BYL719 (Supplementary Table S1).

Induction of cell death was also observed, and the minimum concentrations able to induce cell death were 100 nmol/L BEZ235, 1 µmol/L BKM120, and 5 µmol/L BYL719 (Fig. 1C). It is worth noting that only BKM120 significantly induced cell death at the IC50 concentration. The cytotoxic effect never exceeded 20% with the exception of the effect induced by the highest concentration of BKM120 (10 µmol/L), which is presumably related to off-target effects of the drug (Supplementary Fig. S2).

We then analyzed the modulation of PI3K–AKT–mTOR and MAPK pathways in SKMES-1 and PIK3CA amplified/mutated clones. Dose–response experiments showed that BEZ235 inhibited the phosphorylation of AKT(Ser473), mTOR, and p70S6K and 4E-BP1, mTORC1 downstream substrates, irrespectively of PIK3CA status (Fig. 1D, Supplementary Tables S2–S4). Interestingly, the phosphorylation level of AKT(Thr308) was increased at the highest concentration of the drug (50 nmol/L), and also p-ERK1/2 levels were increased after BEZ235 treatment. These changes were presumably a consequence of the release of mTORC1-mediated inhibitory feedback loop, that triggers PI3K/AKT and MAPK signaling (28, 29). A BEZ235-mediated inhibition of a negative feedback loop operated through mTORC2 might also be involved, as recently suggested (30).

Treatment of SKMES-1 cells and clones with the specific PI3K inhibitors BKM120 and BYL719 induced a similar dose-dependent downregulation of PI3K–AKT–mTOR signaling, as indicated by the reduced phosphorylation of AKT both at Ser473 and Thr308, p70S6K, and 4E-BP1, without any compensatory activation of MAPK pathway. Actually, BYL719 showed an inhibitory activity also on the MAPK pathway.

The dose–response experiments described in Fig. 1D and Supplementary Tables S2–S4 pointed out a different inhibitory action between the specific PI3K inhibitors BKM120 and BYL719 and the dual PI3K/mTOR inhibitor BEZ235. In particular, we observed that BEZ235 inhibited in a dose-dependent manner both p70S6K and 4E-BP1, confirming its main role, at least at the concentrations used in our experiments, as mTOR inhibitor. On the other hand the specific PI3K inhibitors mainly reduced AKT phosphorylation both at Ser473 and Thr308 in a dose-dependent manner.

A time course experiment was then performed to assess the effects of the drugs at their IC50 concentration. As shown in Supplementary Fig. S3, the downregulation of the PI3K–AKT–mTOR pathway observed after 6 hours of drug treatment was
maintained until 48 hours at least in SKMES-1, Cl 10 and Cl 18. In clone 28, the inhibitory effects on p70S6K and 4EBP1 phosphorylation were less evident after 48 hours of treatment.

BEZ235, BKM120, and BYL719 impaired migration/invasion of PIK3CA amplified/mutated cells

Because PtdIns(3,4,5)P³ production and PI3K activity control polarization and migration during chemotactic movement, we evaluated the migration and invasion capability of PIK3CA transfected clones in comparison with SKMES-1 cells. With respect to SKMES-1, the clones showed an enhanced mobility and invasive-ness associated with an increased activity of the matrix metalloproteinases (MMPs) 2 and 9 (Fig. 2A).

Drug treatments significantly reduced cell migration and the minimum concentrations of drugs able to reduce cell migration/invasion capability were 0.1 µmol/L of BEZ235, 1 µmol/L BKM120, and 5 µmol/L BYL719 for 72 hours. Cell death was evaluated by fluorescence microscopy on Hoechst/PI-stained cells. Data are expressed as the percentage of values. D, SKMES-1 cells and indicated clones were treated with vehicle or increased concentration of BEZ235 (0.1 and 1 µmol/L), BKM20, and BYL719 (0.1 and 1 µmol/L). After 6 hours, cells were lysed and indicated protein levels were quantified by Western blotting analysis. Experiments in A and C are the mean value of three independent measurements (±SD). Results in B and D are representative of three independent experiments. *** P < 0.001; ** P < 0.01; * P < 0.05.

The PI3K pathway is involved in the increased cell migration/invasion capability observed in PIK3CA amplified/mutated clones. BKM120 and BYL719 more effectively inhibited cell migration and invasion compared with the dual inhibitor BEZ235 in the range of concentrations near to IC50 for proliferation. Finally, as representatively shown for clone 10, the activity of MMPs was reduced after drug administration (Fig. 2B).

The PI3K–AKT–mTOR pathway is significantly involved in the regulation of cell motility and cytoskeletal rearrangement through the activation of the Rho family of small G proteins, including RhoA, Rac1, and CDC42, via PI3K or mTORC2 (31). Thus, we evaluated by pull down assay the activation status of these proteins in SKMES-1 cells and in PIK3CA amplified/mutated clones. As shown in Fig. 2C, the levels of active GTP-bound CDC42 and RhoA were increased in all clones with respect to SKMES-1 cells, whereas active GTP-bound Rac1 increased in clones 10 and 18. BEZ235 and BKM120 treatments reduced the activation of RhoA, CDC42, and Rac1 in clone 10, whereas...
BYL719 reduced only the activation of RhoA. Similar results were obtained for the other clones (not shown).

The increased migration/invasion capability of PIK3CA amplified/mutated clones prompted us to investigate the expression of a number of proteins involved in EMT. As shown in Fig. 2D, the expression of Vimentin (EMT marker) was increased in clones as compared with SKMES-1 cells whereas E-cadherin, mainly expressed by epithelial cells, was decreased. Moreover, the expression of the transcription factors SLUG and SNAIL, which negatively regulate E-cadherin, augmented in clones with respect to SKMES-1 cells. Finally, b-catenin levels were comparable among all the cells. Interestingly, N-Cadherin expression was detectable only in clone 10 carrying the amplification of PIK3CA wt gene. Overall, these observations confirmed that the oncogenic driver mutations and amplification of PIK3CA promoted the acquisition of mesenchymal properties in SKMES-1 cells.

BEZ235, BKM120, and BYL719 treatment induced, in a dose-dependent way, the expression of E-cadherin in clone 10 (Fig. 2D) and in the other clones (not shown). Moreover, the levels of N-cadherin, vimentin, and SNAIL decreased upon drug treatments.

Effects of BEZ235, BKM120, and BYL719 on growth and migration of 3D cultures from SKMES-1 and PIK3CA amplified/mutated clones

Drug sensitivity may be affected by the spatial distribution of the cells, as it has been previously reported in studies comparing 2D versus 3D culture models (32). Moreover, recent results highlighted the role of CD133-positive cells, displaying cancer stem cell (CSC) like properties, in tumorigenicity and chemoresistance of lung cancer (33). We developed tumor spheres from SKMES-1 and clones and demonstrated that cells from spheroids showed a significant increased surface expression of CD133 with respect to 2D cultured cells (Supplementary Fig. S5). Moreover, in agreement with data on GRQ from 2D cultures (see Fig. 1A), spheroids from clones had a higher proliferation rate than those from SKMES-1 cells (Fig. 3A). Treatment with PI3K inhibitors significantly reduced the
volume of tumor spheres (Fig. 3B). However, as previously observed in 2D cultures, we failed to observe a statistical difference on PI3K-targeted inhibition of cell proliferation between clones and SKMES-1–derived spheroids.

Traditional methods for the evaluation of tumor cell migration in vitro generally use 2D homogeneous cultures that do not take into account tumor heterogeneity, 3D cell–cell contacts between tumor and/or host cells or interactions with extracellular matrix proteins. In this context, we evaluated cell migration from tumor spheroids by using plates precoated with extracellular matrix components. Spheroids from SKMES-1 and clones were transferred onto gelatin-coated plates in the absence or presence of increasing concentrations of BEZ235, BKM120, or BYL719 and the in vitro disentangling of tumor cells was evaluated 48 hours after treatment. As shown in Fig. 3C, SKMES-1 cell release from spheroids was not inhibited by the three compounds, whereas that of PIK3CA amplified/mutated clones was significantly reduced.

Effects of PI3K inhibitors on proliferation, invasiveness, and signaling transduction pathways in SQCLC cell lines carrying PIK3CA mutations

The evaluation of the effects of PI3K inhibitors was extended to H596 and HCC2450 cells, constitutively harboring the PIK3CA E545K and H1047R point mutation, respectively. The IC50 values for BEZ235, BKM120, and BYL719 were comparable with those previously shown for SKMES-1 cells and PIK3CA-transfected clones (Supplementary Table S5). Only BKM120 and BYL719 at the IC50 concentration were able to induce cell death in H596 cells (Fig. 4A). As previously reported for SKMES-1 and clones, the inhibition of mTOR in H596 cells after BEZ235 treatment released the negative regulatory loop with the consequent increased phosphorylation of AKT(Thr308) and ERK1/2. BKM120 and BYL719 exposure downregulated the members of the PI3K–AKT–mTOR pathway in a dose–dependent manner, as indicated by the reduced phosphorylation levels of AKT (both at Ser473 and Thr308 residues) and p70S6K (Fig. 4B).

As previously demonstrated for PIK3CA-transfected clones, both migration and invasion capabilities of H596 and HCC2450 cells were significantly impaired by pure PI3K inhibitors in the range of concentrations near to IC50 for proliferation, whereas BEZ235 was not effective in reducing invasion capability (Fig. 4C and D).

In vivo effects of BYL719 treatment in nude mice carrying SKMES-1 or PI3K-mutated clone xenografts

Finally, we tested in vivo the antitumor effect of BYL719, a specific inhibitor of PI3K p110α subunit. To this purpose, we generated SQCLC xenografts in athymic mice by s.c. injection of PIK3CA-transfected clone 18 or parental SKMES-1 lines. Cells (5 × 106) were injected into the right flank of each mouse and when tumors reached an average volume of 150 mm3, animals were randomly assigned to control or treated (BYL719 30 mg/kg/d) groups and tumor growth was monitored. During the four weeks of treatment, mice showed no signs of toxicity and regularly gained body weight (data not shown). Although transfected cells displayed a higher proliferation rate compared with parental cells in vitro, no difference was detected in tumor growth rate between SKMES-1 and clone 18 xenografts. BYL719 administration significantly affected tumor growth compared with control, both in SKMES-1 and clone 18 xenografts starting from day 20 (Fig. 5A), resulting in a 32% and 38% reduction of tumor volume, respectively, at sacrifice.

Because volume determination in subcutaneous tumor xenografts by caliper diameter did not allow to evidence differences between SKMES-1 and clone 18 xenografts, a more extended histologic analysis was performed revealing some important alterations (34). Indeed, advanced imaging techniques, including CT, PET, and MRI, have intrinsic limitations in detecting and monitoring the in vivo response to treatment of lung cancer experimentally (35) and clinically (36, 37). Therefore, we assessed the therapeutic effect of PI3K inhibitors by accurate morphometric analysis of tumor composition. By this approach, compared with the effect on parental SKMES-1, BYL719 treatment produced a 2-fold increased necrotic tissue in clone 18 xenografts (Fig. 5B and C). Thus, in the presence of similar overall tumor volume, the actual fraction of intact SQCLC cells within BYL719-treated clone 18 xenografts was reduced by 28%, compared with SKMES-1, by specific PI3K inhibition (Fig. 5D).

In addition, to investigate whether the in vitro documentation of the involvement of PI3K pathway on EMT had an in vivo counterpart, the expression of CK7 and VIM was assessed on treated and untreated tumor xenografts by immunohistochemistry. Neoplastic cells carrying the epithelial- or the mesenchymal-associated antigens were clearly distinguishable by double immunostaining (Fig. 6B–E). Interestingly, the proportion of VIMpos neoplastic cells was significantly higher in untreated clone 18 xenografts compared with SKMES-1, suggesting a spontaneous activation of EMT in E545K PIK3CA–mutated tumors (Fig. 6A). BYL719 treatment restored the proportion of CK7pos and VIMpos cells composing clone 18 neoplastic nodules to that of SKMES-1, that conversely was not affected by the drug (Fig. 6A). Thus, PI3K inhibition favors E-cadherin–mediated stabilization of cell adhesions and attenuates the acquisition of a mesenchymal fate in our model of PIK3CA-mutated SQCLC.

Discussion

PI3K–AKT–mTOR represents a key signaling pathway involved in the control of cell proliferation, survival, differentiation, and motility. Importantly, deregulation of several components of this pathway occurs with high frequency in SQCLC.

To test the hypothesis that PIK3CA gene alterations represent an attractive target for SQCLC treatment, we evaluated the effects on cell viability, apoptosis, migration, and invasion of three different PI3K and PI3K/mTOR–targeting agents in SQCLC cell clones, with the overexpressed or mutated PIK3CA gene. The two most commonly mutated sites in p110α subunit of PI3K, E545K in the helical domain and H1047R in the kinase domain (38) were investigated.

One of the major findings of our study was the documentation that, in addition to a common effect on cell proliferation and growth, PI3K inhibitors affect the migratory/invasive and EMT phenotype in SQCLC cells characterized by PIK3CA gene amplification or mutations.

PI3K signaling was increased in clones harboring PIK3CA amplification as well as in cells expressing the p110α E545K– or H1047R-mutated subunits and was associated with an increased proliferation rate, confirming that these alterations may contribute to a more aggressive phenotype (39). PI3K
inhibitors reduced cell proliferation and induced apoptotic cell death to a similar extent in all tested cancer cells, independently from PIK3CA mutation status. The lack of correlation between the baseline activity of the PI3K–AKT–mTOR pathway and the inhibitory activity on cell viability of PI3K-targeted agents has been described in other models such as breast cancer and glioblastoma cell lines (40, 41) as well as in head and neck cancer cells harboring p53 mutated or wt gene (42). In contrast and differently from our results, the activity of PI3K inhibitors on cell viability and induction of cell death has been related to PI3K status in colon cancer cells (43) and in head and neck cancer cells (44). 

In addition, in another article on head and neck cancer cell lines, PIK3CA-mutated cells were sensitive to pathway inhibitors, whereas PIK3CA-amplified cells failed to show sensitivity (45). All together these data indicate that the correlation between PIK3CA status and sensitivity to PI3K inhibitors is still controversial.

BEZ235 was more effective than BKM120 and BYL719 in inhibiting cell proliferation in all tested cell lines both in 2D and in 3D culture. The different sensitivity to PI3K inhibitors is likely attributed to the different mechanism of action of these agents. The dual PI3K/mTOR inhibitor BEZ235 showed a different concentration-dependent behavior toward its targets. At lower concentration BEZ235 strongly inhibits both mTORC1 and mTORC2 complex (IC50 reported for mTOR kinase inhibition is around 6.5 nmol/L) whereas the inhibition of p110α subunit of PI3K occurs at higher concentration of the drug (IC50 29 nmol/L; ref. 46). This different inhibitory activity of BEZ235 resulted in downregulation of mTORC1/2 targets p70S6K, 4EBP1, and AKT(Ser473) in the...
presence of low concentration of the drug (50 nmol/L). Under this condition, mTORC1 inhibition released the negative feedback loop on PI3K/AKT and MAPK via IRSs with the consequent increased phosphorylation of AKT(Thr308) and ERK1/2 (29). This phenomenon was observed in SKMES-1 and H596 squamous lung cancer cell lines as well as in \textit{PIK3CA} amplified/mutated clones after BEZ235 treatment. On this regard, an enhanced MEK/ERK activation has been recently reported in pancreatic cancer cells treated with doses of BEZ235 that inhibited mTORC2-mediated AKT phosphorylation on Ser473, probably due to the suppression of an additional negative feedback loop operated through mTORC2 (30).

On the other hand, pure PI3K inhibitors, as BKM120 and BYL719, were effective at higher concentration than BEZ235, however, prevented the activation of the feedback IRSs-mediated loop, blunting AKT/mTOR signaling.

The results obtained by these experiments confirmed the different activity of the compounds, in particular BEZ235 acts as an mTOR inhibitor with partial effect on AKT, whereas BKM120 and BYL719 by inhibiting full AKT activation were able to reduce invasiveness properties, which are mainly regulated by PI3K.

Increased tumor aggressiveness has been associated with epithelial–mesenchymal transition (EMT), which enhances motility and may confer stem-like properties to cancer cells. EMT is a complex process associated with changes in gene expression, which leads to a dramatic shift of epithelial cells toward the mesenchymal phenotype. PI3K proteins control both cell migration, through the activation of the Rho family of small GTP-ase (47), as well as the transition toward the mesenchymal phenotype (EMT). In this regard, it has been recently reported that PI3K expression is upregulated in mesenchymal tumors of the lung (48) and that oncogenic driver mutations of PI3K promote EMT and cell invasion in breast cancer cells (49).

Our investigation shows that persistent activation of PI3K increased the level of GTP-bound Rho family proteins (RhoA, Rac1, and CDC42) that were downregulated upon treatment with PI3K inhibitors. Importantly, overexpression of Rac1 in NSCLC patients has been associated with poor differentiation, high TNM stage and lymph node metastasis (50).

Along this line of investigation, we demonstrated that hyper-activation of the PI3K–AKT–mTOR pathway, by means of \textit{PIK3CA} copy-number gain or mutations, is involved in the acquisition of a more aggressive behavior of SQCLC. Indeed, \textit{PIK3CA} amplified/mutated clones showed an increased migration and invasion capability as well as an increased expression of mesenchymal markers such as Vimentin, the transcriptional factors Slug and

Figure 5.
Antitumor activity of BYL719 on SKMES-1 and clone 18 tumor xenograft. A, SKMES-1 and clone 18 cells were implanted s.c. on BALB/c-Nude mice. Vehicle or BYL719 (30 mg/Kg) were daily administered by orogastric gavage. Data are expressed as the percentage of change in tumor volume ± SEM of 5 mice per group. (*, P < 0.1; **, P < 0.01; ***, P < 0.001 vs. SKMES-1; #, P < 0.1; ###, P < 0.001 vs. clone 18; two-way ANOVA followed by Bonferroni post-test). H&E-stained sections of BYL719-treated tumors showing gross preservation of squamous epithelial features in SKMES-1 whereas a large necrotic area is apparent in clone 18 (B). The percentage of necrotic area (C) and neoplastic tissue (D) after BYL719 treatment in clone 18 compared with SKMES-1 xenografts; *, P < 0.05.
Snail, and N-cadherin with respect to SKMES-1 cells. In this scenario, a recent work suggests that, although NSCLC cells need the PI3K pathway for proliferation in both the epithelial and mesenchymal states, EMT makes cells more dependent on PI3K pathway activation by enhancing signaling from p110α (48).

The present study demonstrates that PI3K-targeting drugs effectively inhibit cell motility (both in 2D and in 3D culture) and revert the mesenchymal/invasive phenotype. However, the different classes of PI3K inhibitors display a different ability to elicit these effects, in the range concentrations able to inhibit cell proliferation, with pure PI3K target agents BKM120 and BYL719 showing more robust activity against amplified/mutant PIK3CA clones. These distinctive pharmacologic properties may be due to the lower affinity of BEZ235 for PI3K. In fact, our results suggest that PI3K plays a key role in the acquisition of a metastatic phenotype, and that its inhibition may represent the optimal

Figure 6. Effects of BYL719 treatment on SKMES-1 or clone 18 induced tumor xenografts. A, the percentage of CK7- and VIM-positive cells in untreated or BYL719-treated SKMES-1 and clone 18 xenografts (\(\chi^2\), \(P < 0.05\) vs. SKMES-1; \(*\), \(P < 0.05\) vs. untreated). B-E, double immunohistochemical detection of CK7- (brownish) and VIM (pinkish)-positive cells in sections of untreated SKMES-1 (B) and clone 18 (C) tumors. Corresponding BYL719-treated tumors are shown in D and E, respectively. Black rectangles, microscopic fields at higher magnification (B1 and C1, control; D1 and E1, BYL719 treated). Asterisks, E and E1, necrotic areas; scale bars, B-E, 1 mm; B1-E1, 100 μm.
invasion capability of the cells.

In vitro experiments confirmed that tumors from clone 18 retained the mesenchymal phenotype and proved the ability of BYL719 in reducing the expression of Vimentin in Cytokeratin 7–positive cells. Moreover, analysis of tumor composition showed a significant increase of necrotic area and a decrease of the neoplastic tissue in clone 18 respect to SKMES-1 xenografts, thus indicating that tumors carrying PIK3CA E545K mutation were more sensitive to BYL719 treatment.

The metastatic role of PI3K alteration in SQCLC has been recently underlined in a clinical study performed on stage IV SQCLC patients: The hyperactivation of the PI3K pathway resulted in poor survival and was associated with increased metastasis, suggesting that PI3K pathway activation confers a distinct tumor biology (51). Our findings strongly suggest that pharmacologic modulation of PI3K may inhibit EMT mediated cell migration and tumor progression in SQCLC warranting further clinical development of agents such as BKM120 and BYL719 in this lung cancer histologic subtype.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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