

PIK3CA Mutations and Copy Number Gains in Human Lung Cancers

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Abstract

We investigated the frequency and function of mutations and increased copy number of the *PIK3CA* gene in lung cancers. *PIK3CA* mutations are one of the most common gene changes present in human cancers. We analyzed the mutational status of exons 9 and 20 and gene copy number of *PIK3CA* using 86 non-small cell lung cancer (NSCLC) cell lines, 43 small cell lung cancer (SCLC) cell lines, 3 extrapulmonary small cell cancer (ExPuSC) cell lines, and 691 resected NSCLC tumors and studied the relationship between *PIK3CA* alterations and mutational status of epidermal growth factor receptor (EGFR) signaling pathway genes (*EGFR*, *KRAS*, *HER2*, and *BRAF*). We also determined *PIK3CA* expression and activity and correlated the findings with effects on cell growth. We identified mutations in 4.7% of NSCLC cell lines and 1.6% of tumors of all major histologic types. Mutations in cell lines of small cell origin were limited to two ExPuSC cell lines. *PIK3CA* copy number gains were more frequent in squamous cell carcinoma (33.1%) than in adenocarcinoma (6.2%) or SCLC lines (4.7%). Mutational status of *PIK3CA* was not mutually exclusive to *EGFR* or *KRAS*. *PIK3CA* alterations were associated with increased phosphatidylinositol 3-kinase activity and phosphorylated Akt expression. RNA interference-mediated knockdown of *PIK3CA* inhibited colony formation of cell lines with *PIK3CA* mutations or gains but was not effective in *PIK3CA* wild-type cells. *PIK3CA* mutations or gains are present in a subset of lung cancers and are of functional importance. [Cancer Res 2008;68(17):6913–21]

Introduction

Most, if not all, of human malignancies are caused by somatic alterations within the cancer genome, leading to activation of oncogenes or inactivation of tumor suppressor genes, and many of the resultant changes target cell signaling pathways (1). Because of advances in sequencing technology, it is possible to perform exon resequencing for gene families involved in cellular signaling pathways, such as tyrosine kinases, tyrosine phosphatases, and phosphatidylinositol 3-kinases (PI3K; ref. 2). PI3Ks are heterodimeric lipid kinases composed of catalytic and regulatory subunits

encoded by separate genes and subject to alternative splicing (3). The resultant proteins are critical regulators of growth, survival, and motility. PI3Ks are divided into three classes (I–III) according to their substrate preference and sequence homology (4). To date, class IA PI3K α , consisting of catalytic subunit p110 α and regulatory subunit p85 α , is the only PI3K molecule found to have somatic mutations in human cancers; these occur predominantly in helical or kinase domains of its catalytic subunit encoded by the *PIK3CA* gene. Mutations of *PIK3CA* occur in many human epithelial cancers, resulting in *PIK3CA* being one of the two most commonly mutated oncogenes (along with *KRAS*) identified in human cancers (3, 5). However, individual types of epithelial cancers show great variability in their mutational rates, with high rates present in glioblastomas, gastric, hepatocellular, and breast cancers (5), whereas the rates described in non-small cell lung cancers (NSCLC) are relatively low (6, 7). To date, no information is available about small cell lung cancers (SCLC).

In addition to mutations, increased chromosomal copy number (by amplification or polysomy) is another method of oncogene activation (2). In general, increased copy number is frequently associated with increased mRNA expression (8, 9). A region of chromosome 3q (3q25–27), where *PIK3CA* (3q26) is located, is frequently amplified in lung cancers (10), especially squamous cell carcinomas (11, 12). However, the relationship between mutations and amplification of *PIK3CA* has not been studied comprehensively. Also, the functional effects of mutant or amplified *PIK3CA* in lung cancers are still unclear.

The PI3K/Akt pathway lies downstream of certain receptor tyrosine kinases, including epidermal growth factor receptor (EGFR). In addition to *PIK3CA*, other components of the pathway, including loss of the inhibitor PTEN or activating mutations of AKT, occur in certain cancers (13). In lung cancers, mutations have also been reported in several other genes involved in the EGFR signaling pathway, including *EGFR*, *KRAS*, *HER2*, and *BRAF* (14–16). As activation of EGFR family signaling is a mechanism to activate class IA PI3K (either directly or through RAS), we investigated the relationship between mutations of these genes and *PIK3CA* alterations.

As relatively little is known about the role of PI3K deregulation in lung cancer, we documented the frequency of mutations and amplifications in NSCLC tumors from four countries and in NSCLC and SCLC cell lines. We also studied the functional effects on PI3K deregulation in NSCLC cell lines.

Materials and Methods

Cell lines. We studied 86 NSCLC and 43 SCLC cell lines and three extrapulmonary small cell cancers (ExPuSC; ref. 17), most of which were

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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established by the authors (18). We also investigated eight immortalized human bronchial epithelial cell lines (HBEC; refs. 19, 20). NCI-H3255 was obtained from Dr. Bruce Johnson (21). PC-9 (originally from Tokyo Medical University) was obtained from Dr. Bert Vogelstein. Calu-3 was purchased from the American Type Culture Collection.

Tumor samples. We previously reported on the mutational status of *EGFR*, *KRAS*, and *HER2* in NSCLC (14, 15). Of the 617 samples used in that study, DNAs from 591 tumors were available for *PIK3CA* and *BRAF* mutational studies. In this study, we included a further 100 samples obtained from Okayama University. Thus, the total number of tumor samples available for mutational analyses of the five genes was 691. These tumors were from patients undergoing surgical resection from Japan ($n = 323$), Taiwan ($n = 148$), the United States ($n = 150$), or Australia ($n = 70$). Sufficient DNA was available from 356 of these tumors for *PIK3CA* gene copy number analysis. Corresponding nonmalignant adjacent tissues ($n = 267$) were also available. Institutional Review Board permission and informed consent were obtained at each collection site.

For array comparative genomic hybridization (CGH) analysis, microdissected DNA was available from a separate set of 40 fresh-frozen NSCLC obtained from Vancouver General Hospital.

DNA and RNA extraction. Genomic DNA was obtained from primary tumors and cell lines by standard phenol-chloroform (1:1) extraction followed by ethanol precipitation or by using DNeasy Tissue kit (QIAGEN). Total RNA was obtained from cell lines using RNeasy Plus Mini kit (QIAGEN).

Gene mutational analyses. The intron-based PCR primer sequences for *PIK3CA* (exons 9 and 20) with PCR product lengths of 273 and 359 bp, respectively, and PCR conditions are provided in Supplementary Data. Primers were designed to avoid amplification of a known *PIK3CA* pseudogene (22). All sequence variants were confirmed by independent PCR amplifications and sequenced in both directions. With one exception (U1171), all of the mutant cases had corresponding nonmalignant tissue DNA available and were of somatic origin.

BRAF mutational status (exons 11 and 15) was determined, as described in Supplementary Data.

***PIK3CA* copy number evaluation by real-time quantitative PCR.** We studied *PIK3CA* copy number in all of the cell lines, 356 NSCLC tumors, and 267 corresponding nonmalignant adjacent tissues using real-time quantitative PCR, as described in Supplementary Data. Samples were analyzed in triplicate. Each amplification reaction was checked for the absence of nonspecific PCR products by melting curve analysis. *PIK3CA* copy number calculation was carried out using the comparative C_t method (23) after validating that the efficiencies of PCR reactions of both *PIK3CA* and *COL8A1* were equal. Human genomic DNA (EMD Biosciences), which is made from a mixture of pooled human whole blood from six to eight individual male and female donors, was run in every assay as a calibrator sample. *PIK3CA* gene copy number in normal human genomic DNA was set as 2 and copy number of more than 4 in cell lines was considered as increased. For clinical samples, we reduced the cutoff value from 4 to 3 because contamination with nonmalignant cells is invariably present (estimated average per tumor = 50% tumor cells, 50% nonmalignant cells).

Tiling path array CGH. Array CGH was performed as previously described (24). Further details are provided in Supplementary Data.

***PIK3CA* mRNA expression.** We analyzed *PIK3CA* mRNA expression levels in lung cancer cell lines as a part of RT² Profiler Custom PCR Array (SuperArray Bioscience). After making cDNA from 1.0 μ g total RNA using RT² PCR Array First Strand kit (SuperArray Bioscience), quantitative PCR was performed with the Chromo4 PCR System (Bio-Rad Laboratories) using RT² Real-Time SYBR Green PCR Master Mix (SuperArray Bioscience) according to the manufacturer's protocol. We chose three different housekeeping genes, β -actin (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase, and hypoxanthine phosphoribosyltransferase 1, as internal controls, and the averages of their C_t values were used. We also analyzed the values of six HBECs (HBEC3KT, HBEC4KT, HBEC5KT, HBEC17KT, HBEC30KT, and HBEC34KT) for *PIK3CA* mRNA expression, and the tumor cell values were expressed relative to the mean of the six HBECs. *PIK3CA* mRNA relative value for each HBEC cell line was also calculated by comparison to the

average value of the six HBECs. For data analysis, the comparative C_t method (23) was used.

PI3K activity assay. PI3K activity of lung cancer cell lines was measured using an ELISA kit (Echelon Biosciences)¹¹ according to the manufacturer's protocol. PIP₃ produced by one of *PIK3CA* wild-type cell line with normal copy number (NCI-H1299) was set as 100 (control), and others were compared with the control and expressed as relative values.

Western blot analysis. Preparation of total cell lysates and Western blotting were done as described previously (20). Primary antibodies used were rabbit polyclonal anti-PI3K p110 α (Cell Signaling), rabbit polyclonal anti-Akt (Cell Signaling), rabbit polyclonal anti-phosphorylated Akt (pAkt; Ser⁴⁷³; Cell Signaling), rabbit polyclonal anti-PTEN (Cell Signaling), rabbit polyclonal anti-phosphorylated PTEN (Ser³⁸⁰; Cell Signaling), and mouse monoclonal anti-actin (Sigma-Aldrich) antibodies. Actin levels were used as a control for protein loading. Peroxidase-labeled antirabbit or antimouse antibodies (Amersham Pharmacia) were used as the second antibody.

RNA interference studies. RNA interference of *PIK3CA* was performed by two methods, using either small interfering RNA (siRNA) or small hairpin RNA (shRNA). For each technique, two individual sequences targeting different regions of the gene were used as described below.

Preparation and transfection of siRNAs. siRNAs targeting *PIK3CA* were designed through the RNAi Co. Ltd. website¹² based on siDirect online software system (25), which can efficiently select siRNA sequences avoiding off-target effects. siRNA sequences targeting two different regions of *PIK3CA* are provided in Supplementary Data. The siRNA sequences against *Tax* (the human leukemia virus gene) were reported previously (26). The siRNAs were chemically synthesized by Dharmacon. Cells were transfected with 5 nmol/L of siRNA using HiPerFect Transfection Reagent (QIAGEN) according to the manufacturer's protocol. Control cells were treated with HiPerFect alone or with *Tax* siRNA. Cells were grown and harvested 72 h after the transfection for additional analyses.

Preparation and transfection of shRNAs. HuSH 29mer shRNA constructs against *PIK3CA* (TR310428) gene-specific shRNA expression plasmids, along with control shRNA plasmids, including the original pRS vector (TR20003), were purchased from OriGene. We used the following shRNAs: pRSPIK3CA_1 (TI341705) and pRSPIK3CA_3 (TI341707). To produce viral-containing medium, 293T cells were transiently transfected with shRNA vectors together with pVpack-VSVG and pVpack-GP vectors (Stratagene). Transfection assays were performed as described previously (20). Two days after incubation, drug selection was initiated with 2 μ g/mL of puromycin.

Colony formation assays. The *in vitro* growth characteristics were tested by colony formation assays. siRNA-transfected cells were harvested 72 h after the transfection and colony formation assays were performed. For shRNA experiments, shRNA-transfected cells were selected appropriately by antibiotics, and the colony formation assays were performed. For liquid colony formation assays, 500 viable cells were plated onto six-well plates in triplicate. Cells were cultured in RPMI 1640 supplemented with 5% serum, and colonies were counted 14 d later after staining with 0.5% methylene blue. For soft agar growth assays, 2,000 viable cells were suspended and plated in 0.33% agar in appropriate medium with 10% fetal bovine serum and layered over a 0.50% agar base in six-well plates in triplicate. After 2 wk, the number of microscopically visible colonies (>50 cells) was counted.

Statistical analyses. All statistical analyses in this study were performed using GraphPad Prism 4 software (GraphPad Software).

Results

***PIK3CA* mutations in lung cancer cell lines and tumors.** We analyzed *PIK3CA* mutations in exons 9 and 20 in lung cancer cell lines and tumors (Supplementary Fig. S1; Tables 1 and 2) because

¹¹ <http://www.echelon-inc.com/>

¹² http://www.rnai.co.jp/e_index.html

Table 1. Summary of *PIK3CA* mutations in lung cancers

Exon	Amino acid change	Functional domain	NSCLC cell lines (n = 86)	NSCLC tumors (n = 691)	SCLC cell lines (n = 43)	ExPuSC cell lines (n = 3)	Total no. mutations
9	S541F	Helical	0	1*	0	0	1
9	E542K	Helical	0	2*	0	0	2
9	E545K	Helical	2	5	0	0	7
9	Q546K	Helical	1	0	0	0	1
20	M1043L	Kinase	0	1	0	0	1
20	H1047R	Kinase	1	1	0	1	3
20	H1047L	Kinase	0	2	0	1	3
Total cell lines/tumors (%)			4 (4.7%)	11* (1.6%)	0 (0%)	2 (66.7%)	

*One NSCLC tumor had both S541F and E542K mutations. For histologic subtype data of cell lines, see Table 2. Mutations in the 691 NSCLC tumors occurred in the following histologic subtypes: 5 of 249 (2.0%) squamous cell tumors, 5 of 400 (1.3%) adenocarcinomas, and 1 of 42 (2.4%) other NSCLC tumors.

80% of the reported mutations of *PIK3CA* are clustered in these two exons (27). We identified *PIK3CA* mutations in NSCLC cell lines (4 of 86, 4.7%) and tumors (11 of 691, 1.6%; Table 1). *PIK3CA* mutations were distributed among all the major histologic subtypes (Table 1 and Supplementary Tables S1 and S2). Mutations in cancer cell lines of small cell origin were limited to two of three ExPuSC cell lines (Table 1 and Supplementary Table S2). All of these mutations were missense mutations. One primary tumor (U1171) had two missense mutations and one of these (C1622T, S541F) was a novel sequence variant (Supplementary Fig. S1). Whereas mutations at M1043 position have been previously described (27), an A3127C (M1043L) mutation, detected in tumor J-OK398, was also a novel sequence variant (Supplementary Fig. S1). The mutations in NSCLC were more frequent in exon 9 (3 of 4 in cell lines and 7 of 11 in tumors) than in exon 20, whereas both mutations in ExPuSC cell lines were in exon 20.

The frequencies of mutations in these four genes in 86 NSCLC cell lines were as follows: *EGFR* (n = 10, 11.6%), *KRAS* (n = 26, 30.2%), *HER2* (n = 1, 1.2%), *BRAF* (n = 5, 5.8%). There were no

mutations in these four genes in 35 SCLC cell lines. We confirmed that the mutational status of *EGFR*, *KRAS*, *HER2*, and *BRAF* in NSCLC tumors and cell lines were mutually exclusive. However, we found that one cell line and three clinical samples with *PIK3CA* mutations also had *KRAS* or *EGFR* mutations (Supplementary Table S3). Thus, whereas mutations in the other EGFR pathway genes (*EGFR*, *KRAS*, *HER2*, and *BRAF*) are mutually exclusive, mutations of *PIK3CA* are not mutually exclusive to these genes.

Relationship of mutations and clinicopathologic features. A summary of the relationship between mutational status of NSCLC tumors and clinicopathologic features is presented in Supplementary Table S4 (individual tumor information is presented in Supplementary Table S1). As indicated by our data and several previous reports (14, 15), *EGFR*, *KRAS*, and *HER2* mutations target specific histology, gender, smoking status, and ethnic subgroups. Our data confirm these findings and extend them to *BRAF* mutations (although the data are limited). However, *PIK3CA* mutations do not show association with any of these clinicopathologic features.

Table 2. *PIK3CA* mutation or copy number gain in lung cancer cell lines and tumors

Samples	No. samples with the following factor in <i>PIK3CA</i>			
	Mutations	Copy number gain	Both mutations and gain	Either mutations or gain
NSCLC cell lines (n = 86)	4 (4.7%)	8 (9.3%)	1 (1.2%)	11 (12.8%)
Squamous cell lines (n = 14)	1 (7.1%)	5 (35.7%)	1 (7.1%)	5 (35.7%)
Adenocarcinoma cell lines (n = 51)	1 (2.0%)	3 (5.9%)	0 (0%)	4 (7.8%)
Other NSCLC cell lines (n = 21)	2 (9.5%)	0 (0%)	0 (0%)	2 (9.5%)
NSCLC tumors (n = 356)	11 (3.1%)*	61 (17.1%)	4 (1.1%)	68 (19.1%)
Squamous cell tumors (n = 139)	5 (3.6%)	46 (33.1%)	3 (2.2%)	48 (34.5%)
Adenocarcinomas (n = 195)	5 (2.6%)	12 (6.2%)	0 (0%)	17 (8.7%)
Other NSCLC tumors (n = 22)	1 (4.5%)	3 (13.6%)	1 (4.5%)	3 (13.6%)
SCLC cell lines (n = 43)	0 (0%)	2 (4.7%)	0 (0%)	2 (4.7%)
ExPuSC cell lines (n = 3)	2 (66.7%)	0 (0%)	0 (0%)	2 (66.7%)

**PIK3CA* mutations were detected in 11 of 691 NSCLC tumors (1.6%), as shown in Table 1. However, as only 356 of 691 tumors were analyzed for both mutations and gene copy number of *PIK3CA* (all 11 mutant tumors were represented in this subset), the frequency of mutations in this subset increased to 3.1%.

PIK3CA copy number evaluation in lung cancer cell lines and tumors. We evaluated *PIK3CA* copy number by real-time quantitative PCR (Fig. 1). Increased *PIK3CA* copy number was detected in eight (9.3%) of NSCLC cell lines, two (4.7%) of SCLC cell lines, none of three ExPuSC lines, and in 61 (17.1%) of NSCLC tumors. Increased *PIK3CA* copy number was significantly more frequent in squamous cell carcinomas (5 of 14 cell lines, 35.7%, and 46 of 139 tumors, 33.1%) than in adenocarcinomas (3 of 51 cell lines, 5.9%, and 12 of 195 tumors, 6.2%; Fig. 1; Supplementary Tables S1 and S2), $P = 0.009$

and $P < 0.0001$, respectively. Adenocarcinomas with *PIK3CA* copy gain occasionally had other gene mutations (one of three in cell lines and 7 of 12 in primary tumors; see Supplementary Tables S1-S3), whereas squamous cell carcinomas with *PIK3CA* copy gains tended to have other gene mutations less frequently (a single tumor with copy gain also had a *KRAS* mutation).

In both tumors and cell lines, copy gain values were usually modest (<5 copies per cell). With one exception (adenocarcinoma line NCI-H3255, copy number 6.61, also having an *EGFR* mutation),

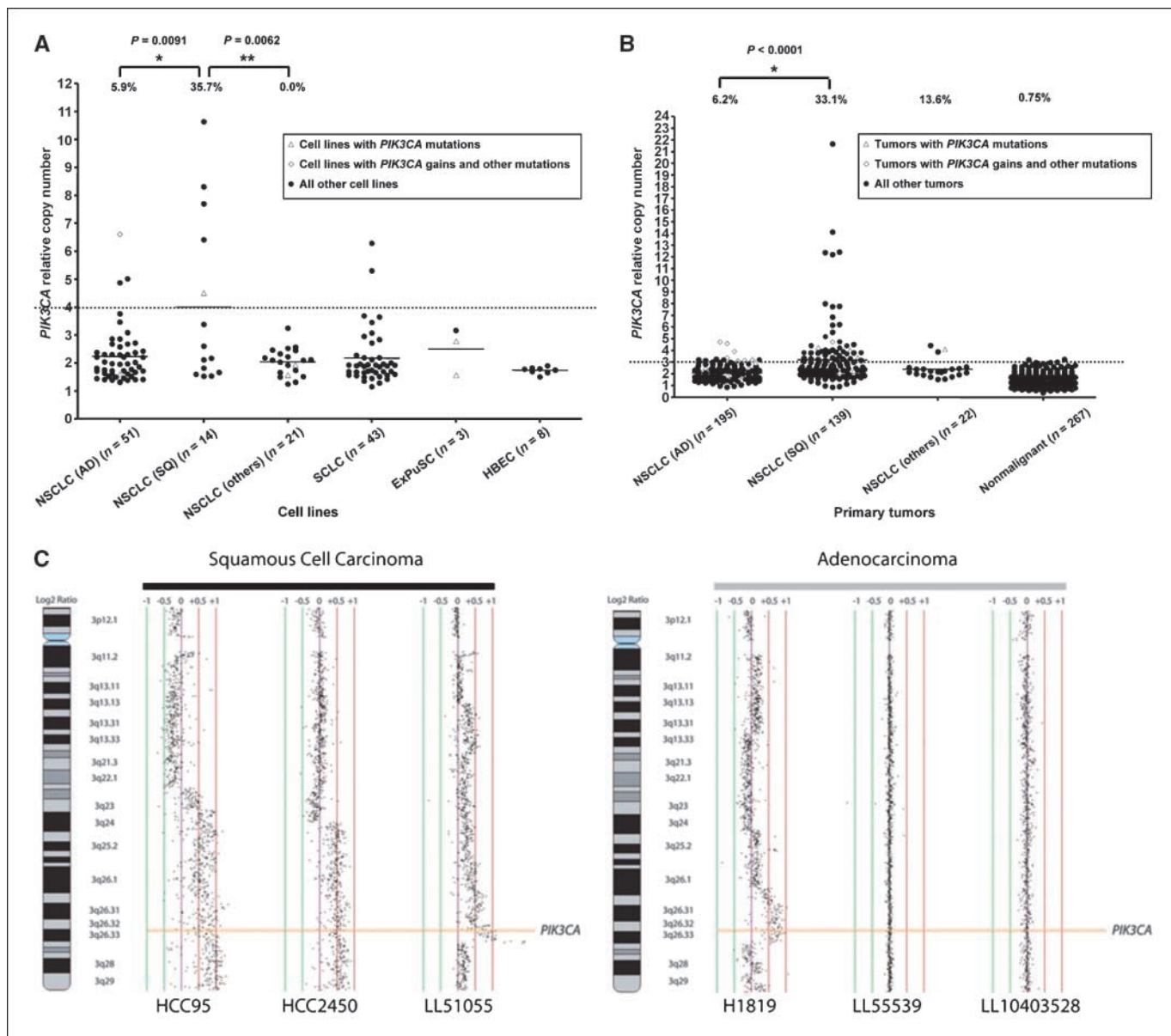
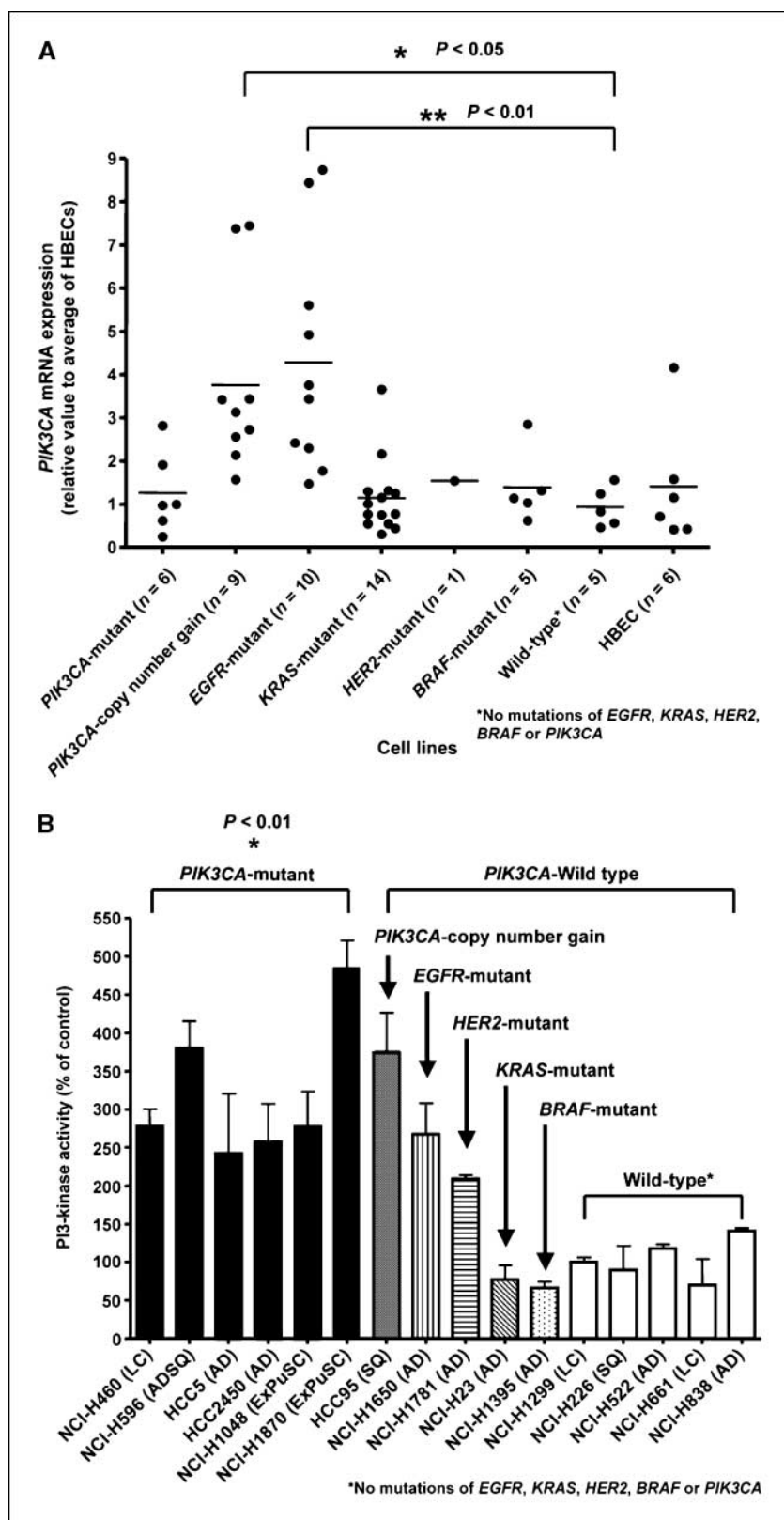


Figure 1. *PIK3CA* gene copy number in lung cancer cell lines (A) and primary tumors (B). Array CGH. Representative array CGH profiles at the *PIK3CA* gene locus for squamous cell carcinoma and adenocarcinoma (C). A and B, *PIK3CA* copy number was expressed as relative values compared with pooled human genomic DNA from multiple healthy donors. A, copy number in lung cancer cell lines. Open triangles indicate *PIK3CA* mutant cell lines, and open diamonds indicate *PIK3CA* copy number gain cell lines with mutations in other EGFR pathway genes. B, copy number in primary lung tumors. Corresponding adjacent nonmalignant tissues ($n = 267$) were also available. Copy numbers more than 4 (in cell lines) or 3 (in tumors) were considered as increased. We reduced the cutoff value to determine gains from 4 (for cell lines) to 3 for tumors to compensate for contamination with nonmalignant tissue in tumor samples (estimated average = 50% tumor cells, 50% nonmalignant cells). Fisher's exact test (cell lines) or χ^2 test (primary tumors) were used to determine significance. C, normalized log₂ signal intensity ratios were plotted using *SeeGH* software. Vertical lines denote log₂ signal ratios from -1 to +1 with copy number increases to the right (red lines) and decreases to the left (green lines) of 0 (purple line). Each black dot represents a single bacterial artificial chromosome (BAC) clone. The region containing *PIK3CA* is shaded orange. For squamous cell carcinomas, two cell lines (HCC95 and HCC2450) displaying long segment *PIK3CA* gain/amplification and a clinical tumor specimen (LL51055) demonstrating focal amplification are depicted. For adenocarcinomas, one cell line, NCI-H1819 (H1819), with long segment high-level gain is depicted along with two clinical tumor specimens with diploid copy number for *PIK3CA*.

Figure 2. *PIK3CA* mRNA expression (A) and PI3K activity (B) in lung cancer cell lines. A, *PIK3CA* mRNA expression was compared among cell lines having different features, such as *PIK3CA* alterations or mutations of other genes involved in the EGFR signaling pathway. *PIK3CA* mRNA expression was expressed as a relative value compared with the mean of values of six HBEC cell lines. *PIK3CA* mRNA expression in *PIK3CA* gain or *EGFR* mutant cell lines was significantly increased compared with that of wild-type cell lines. However, *PIK3CA* mutant lines do not express increased mRNA levels. Horizontal bars indicate mean values. The Kruskal-Wallis test with Dunn's multiple comparison test was used to determine significance. B, equal amounts of protein (500 μg) were immunoprecipitated with anti-PI3K p85 antibody. The PI3K activity of each precipitate was then measured by ELISA and expressed as the relative production of phosphatidylinositol 3,4,5-triphosphate (PIP₃) in each sample using a commercially available ELISA kit. PIP₃ produced by NCI-H1299 cells (being wild-type for *EGFR*, *KRAS*, *HER2*, *BRAF* or *PIK3CA*, *PIK3CA* copy number 2.45) was set as 100 (control), and others were compared with this value. Each assay was done in triplicate. Mean values of PI3K activity in *PIK3CA* mutant cell lines were significantly higher than those of wild-type cell lines. Two cell lines having mutations of *EGFR* or *HER2* also had relatively high PI3K activity, indicating that mutant *HER2* or *EGFR* activates PI3K, which is downstream of these genes. Error bars indicate SD. The Mann-Whitney U test was used to determine significance.



all the NSCLC cell lines and tumors having high-level gains (>5 copies per cell) were squamous cell cancers.

We also examined for geographic differences between East Asian and other ethnicities (mainly Caucasian) in *PIK3CA* copy number.

Although not statistically significant, East Asian tumors tended to have more cases with increased *PIK3CA* copy number in both adenocarcinoma and squamous cell carcinoma compared with other ethnicities. *PIK3CA* copy gains were more frequent in

squamous cell carcinomas than in adenocarcinomas in both ethnicities (41.2% of squamous cell carcinomas and 9.5% of adenocarcinomas in East Asians and 25.4% of squamous cell carcinomas and 1.3% of adenocarcinomas in other ethnicities).

Table 2 indicates the frequencies of either *PIK3CA* mutation or copy gains in lung cancers. *PIK3CA* alterations were more frequent in squamous cell carcinoma than in adenocarcinoma.

To verify the *PIK3CA* copy number results obtained by qPCR, we also examined a subset of cell lines and a separate cohort of tumors by array CGH. Figure 1C shows representative results for *PIK3CA* amplification. We obtained similar results of *PIK3CA* copy number by both techniques in cell lines HCC95, HCC2450, and NCI-H1819 (also see Supplementary Tables S2 and S5). CGH data confirmed that *PIK3CA* copy gains are more frequent in squamous cell carcinomas than in adenocarcinomas from North America (Supplementary Table S6).

***PIK3CA* mRNA expression in lung cancer cell lines.** *PIK3CA* mRNA relative expression in lung cancer cell lines with *PIK3CA* mutation or increased copy number and other mutations was compared with that of wild-type cell lines (Fig. 2A). Wild-type cell lines indicate cell lines without mutations of *EGFR*, *KRAS*, *HER2*, *BRAF*, or *PIK3CA*. *PIK3CA* mRNA expression was significantly increased in cell lines with *PIK3CA* copy gains (*cPIK3CA*) or *EGFR* mutations (*mEGFR*) compared with wild-type cell lines. Eight of 9 *cPIK3CA* and 8 of 10 *mEGFR* cell lines had values more than double the mean values for all tumor lines, whereas lines with mutations other than *mEGFR* (including *mPIK3CA* lines without copy gains) had similar mRNA expression levels. Highly expressed *PIK3CA* mRNA was observed only in *cPIK3CA* or *mEGFR* cell lines.

PI3K activity and protein expression profile in PI3K/Akt pathway in lung cancer cell lines. We measured PI3K activity by competitive ELISA in a subset of 16 cell lines (Fig. 2B). We tested all available *mPIK3CA* cell lines and representative examples of cell

lines with other molecular alterations. The mean value of PI3K activity in all six *mPIK3CA* cell lines was significantly increased compared with that of five *PIK3CA* wild-type cell lines (i.e., wild-type cell lines here means cell lines without mutations of any of the five tested genes). One *cPIK3CA* cell line, one *mEGFR* cell line, and one *mHER2* cell line had increased kinase activity, whereas *mKRAS* or *mBRAF* cell lines had no increased kinase activity. Thus, PI3K activity was increased in lines having *PIK3CA* mutations or increased copy number or by mutations in genes upstream of *PIK3CA*.

We also correlated genotypic changes of *PIK3CA* with protein expression of PI3K pathway genes *AKT* and *PTEN* (Fig. 3). *PIK3CA* mutations and gains were strongly correlated with expression of activated pAkt (Ser⁴⁷³). Phosphorylated PTEN (Ser³⁸⁰), which counteracts *PIK3CA* (28), is expressed by most of lung cancer cell lines.

RNA interference of *PIK3CA*. To investigate the phenotypic effects of *PIK3CA* mutations and gains, we performed RNA interference-mediated knockdown of *mPIK3CA*, *cPIK3CA*, and wild-type cell lines (Fig. 4). As detailed in the legend of Fig. 4, using both transient (siRNA) or stable (shRNA) techniques, expression of both PI3K and its downstream effector molecule AKT could be down-regulated in both *mPIK3CA* and *cPIK3CA* cell lines (only the data of representative siRNA experiments are shown). RNA interference also inhibited PI3K expression in a wild-type line having a near diploid gene copy number (NCI-H226). However, this cell line was the only line that did not express pAKT in its unaltered state.

As illustrated in Fig. 4, RNA interference-mediated knockdown of PI3K inhibited colony formation in both anchorage-dependent and anchorage-independent conditions in both *mPIK3CA* and *cPIK3CA* cell lines but had no effect on the growth of the wild-type cell line with near diploid copy number (NCI-H226). Of the lines tested, one had a mutation in exon 9 and one in

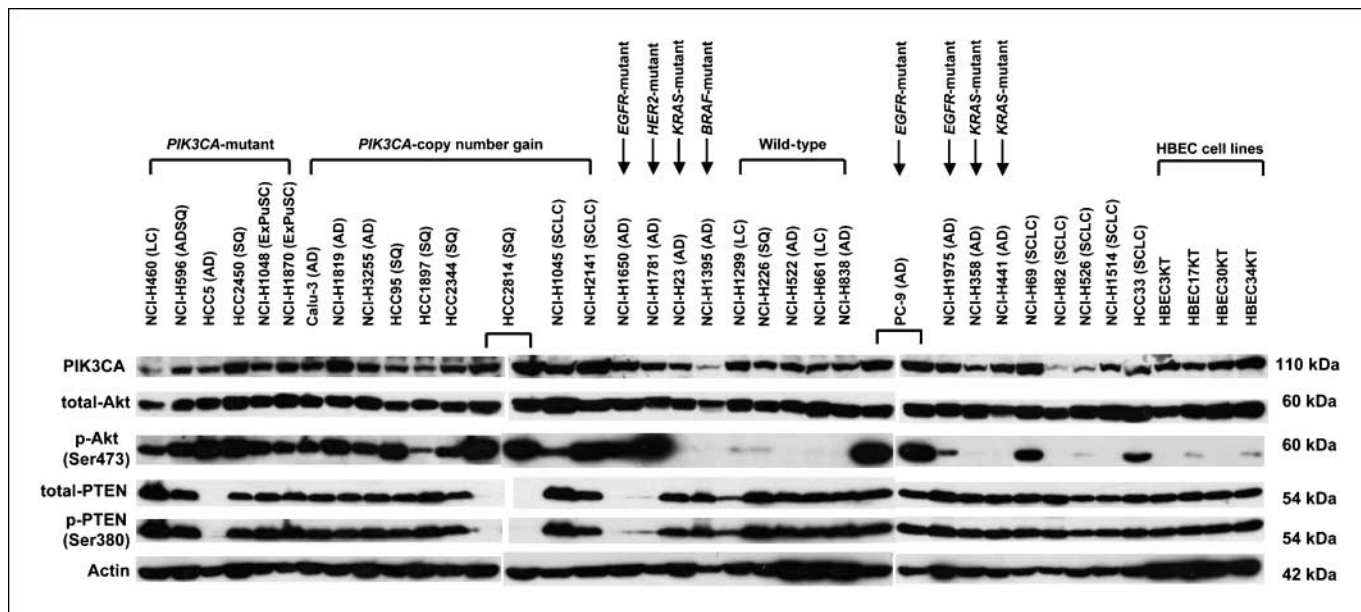


Figure 3. Protein expression profiles of PI3K/Akt pathway genes in lung cancer cell lines. Whole-cell lysates were subjected to Western blotting using the antibodies indicated in the left-hand column. Total PIK3CA is expressed in all lung cancer cell lines to varying degrees. However, pAkt, which is downstream of PIK3CA, is expressed in all the *PIK3CA* mutant and *PIK3CA* high copy number cell lines. pAkt was also detected in *EGFR* mutant and *HER2* mutant cell lines but was not detected in *KRAS* mutant or *BRAF* mutant cell lines. Of interest, high expression of pAkt is present in some SCLC lines lacking known *EGFR* pathway alterations. PTEN, which counters PI3K activity, is phosphorylated (and thus inactivated) at position Ser³⁸⁰ in most of the lung cancer cell lines.

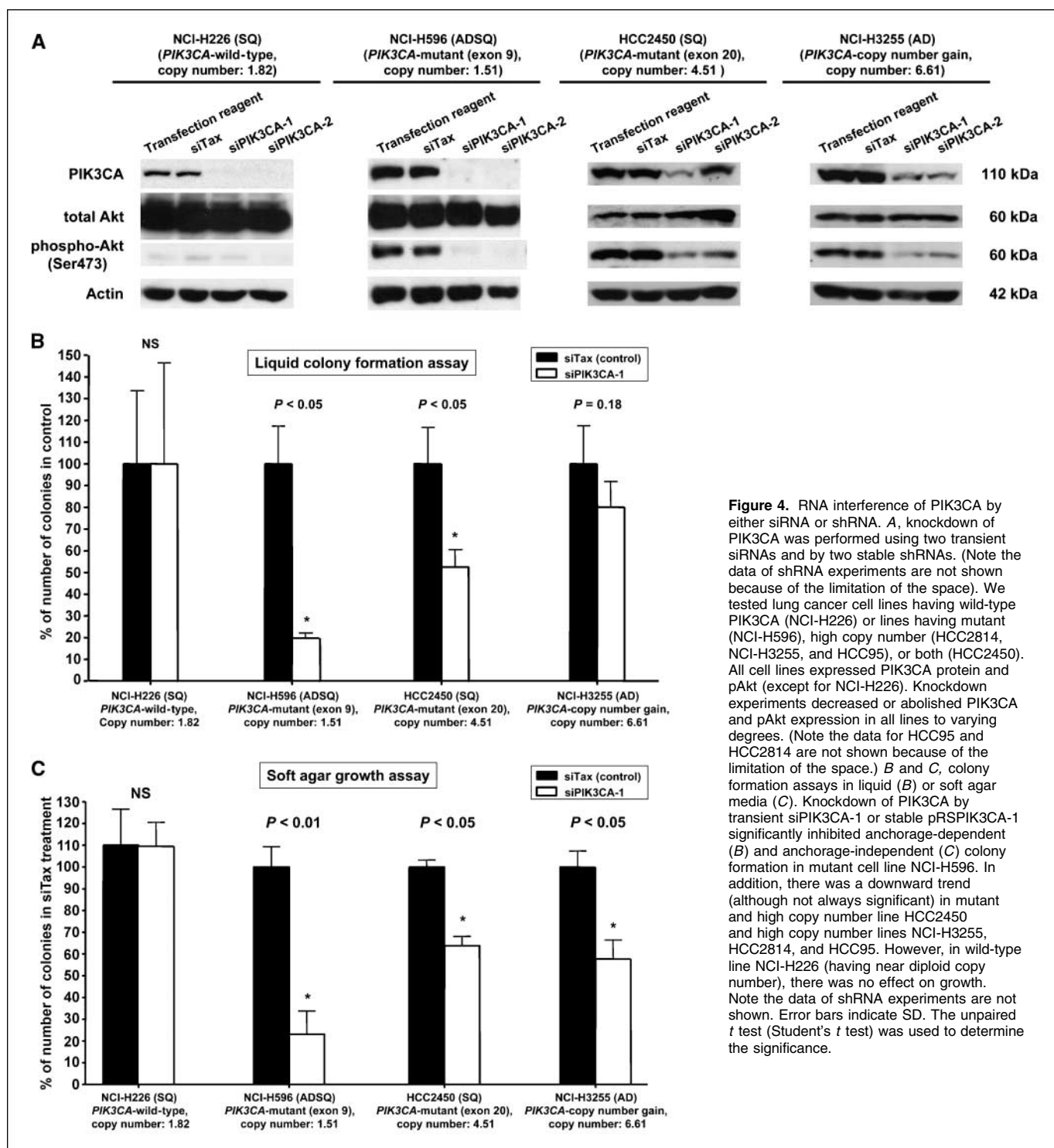


Figure 4. RNA interference of PIK3CA by either siRNA or shRNA. **A**, knockdown of PIK3CA was performed using two transient siRNAs and by two stable shRNAs. (Note the data of shRNA experiments are not shown because of the limitation of the space.) We tested lung cancer cell lines having wild-type PIK3CA (NCI-H226) or lines having mutant (NCI-H596), high copy number (HCC2814, NCI-H3255, and HCC95), or both (HCC2450). All cell lines expressed PIK3CA protein and pAkt (except for NCI-H226). Knockdown experiments decreased or abolished PIK3CA and pAkt expression in all lines to varying degrees. (Note the data for HCC95 and HCC2814 are not shown because of the limitation of the space.) **B** and **C**, colony formation assays in liquid (**B**) or soft agar media (**C**). Knockdown of PIK3CA by transient siPIK3CA-1 or stable pRSPiK3CA-1 significantly inhibited anchorage-dependent (**B**) and anchorage-independent (**C**) colony formation in mutant cell line NCI-H596. In addition, there was a downward trend (although not always significant) in mutant and high copy number line HCC2450 and high copy number lines NCI-H3255, HCC2814, and HCC95. However, in wild-type line NCI-H226 (having near diploid copy number), there was no effect on growth. Note the data of shRNA experiments are not shown. Error bars indicate SD. The unpaired *t* test (Student's *t* test) was used to determine the significance.

exon 20. With minor discrepancies, the results of siRNA and shRNA interference were complementary.

Discussion

The PI3K/Akt pathway is one of the important regulators of mammalian cell proliferation and survival, because several components of this pathway are deregulated in many cancers

(13). We performed a large-scale mutational and copy number gain analysis of lung cancer cell lines and primary tumors followed by functional analyses. Consistent with earlier smaller-scale analyses by others (7, 27), we found a relatively low percentage of PIK3CA mutations in lung cancers compared with several other epithelial cancers. Whereas we did not find PIK3CA mutations in SCLC cell lines of pulmonary origin, we found mutations in two ExPuSC cell lines. To date, mutations in the EGFR pathway genes that are well

studied (*EGFR*, *BRAF*, *KRAS*, *HER2*, and *PIK3CA*) have not been reported in SCLC.

Previously, we reported that mutations of *EGFR*, *KRAS*, and *HER2* were mutually exclusive, indicating that at least one activating mutation in the EGFR-RAS-RAF signaling pathway is sufficient for the pathogenesis of many lung cancers (15). In this study, we added *BRAF* to the mutual exclusion list. However, the mutational status of *PIK3CA* was not mutually exclusive to *EGFR* or *KRAS*, which is similar to previous reports (6, 29, 30). Whereas the other genes targeted adenocarcinomas, *PIK3CA* mutations targeted different subpopulations of NSCLC.

PI3K may be activated by receptor kinases and Ras, which in turn activates Akt. However, whereas the PI3K and EGFR signaling pathways closely interact, PI3K signaling has additional activators and downstream targets (4). Our findings of dual mutations in *PI3KCA* and genes within the direct EGFR signaling pathways are consistent with these observations.

We found that *PIK3CA* copy number gains in cell lines and tumors were more frequent in squamous cell carcinomas than in adenocarcinomas, which is consistent with previous smaller studies (11, 12). Array CGH analyses of a subset of our cell lines and an independent smaller tumor set confirmed the qPCR data. Adenocarcinomas with *PIK3CA* gains tended to have other gene mutations, suggesting that *PIK3CA* gain may not be enough for the pathogenesis of adenocarcinoma. However, most of squamous cell carcinomas with *PIK3CA* gains had no other alterations in the genes studied, indicating that *PIK3CA* may play a pivotal role in pathogenesis of squamous cell cancers. High-level *PIK3CA* gains (copy number, >5) were exclusively present in squamous cell carcinomas. *PIK3CA* mutations and copy number gains occurred independently of each other, and either molecular event may be sufficient to drive the cell toward tumorigenesis.

We profiled *PIK3CA* mRNA expression, PI3K activity and protein expression status in PI3K/Akt pathway to see if *PIK3CA* alterations have a biological effect. Cell lines with *PIK3CA* gains had increased mRNA expression, as did lines with *EGFR* activating mutations, as previously reported (31, 32). Somewhat surprisingly, *PIK3CA* mutant cell lines did not have high mRNA expression.

However, cell lines having either *PIK3CA* mutations or gains (or both) were associated with increased PI3K activity and increased expression of pAkt protein (but not with increased total PIK3CA protein) when compared with wild-type cell lines or immortalized bronchial epithelial cells. *EGFR* and *HER2* mutant cell lines had the same effects, whereas *KRAS* and *BRAF* mutant cell lines did not have these effects. According to previous reports, mutant PIK3CA protein causes a gain of enzymatic function and induces oncogenic transformation when expressed in primary chicken embryo fibroblasts and in NIH 3T3 cells (33, 34). Samuels and colleagues

also reported that mutant PIK3CA promotes cell growth and invasion of human cancer cells (35). Expression of PTEN, a negative regulator of the PI3K/Akt pathway, was present (as total and phosphoprotein) in most of lung cancer cell lines irrespective of their mutational or copy number status.

To determine whether alterations in *PIK3CA* contribute toward the oncogenic phenotype, we used RNA interference, both transient (siRNA) and stable (shRNA). Results of both knockdown techniques were similar and showed reduction of PIK3CA and pAkt proteins in mutant and high copy number cell lines accompanied by decreased anchorage-dependent and independent growth. However, knockdown of PIK3CA protein in a wild-type cell line lacking detectable pAkt had no effect on cell growth. These results confirm that mutations or copy number gains in squamous lung cancer cells confer a growth advantage and that inhibition of pAkt may be a better indicator of cellular effect (and perhaps of therapeutic efficacy) than inhibition of PIK3CA protein.

The finding that PI3K, a key regulator of cell growth, metabolism, and survival, is frequently activated in cancers has stimulated widespread interest in identifying potent and selective inhibitors of PI3K isoforms (36). Whereas several potential therapeutic agents have been identified, most do not show isoform selectivity. Recently, the structure of a human p110 α /p85 α complex was clarified, and the location of the common mutations at specific interfaces identified (37). These new insights into the structure of *PIK3CA* offer insights into the design of specific inhibitors.

In conclusion, *PIK3CA* copy number gains occur at much higher frequencies in lung cancers than do activating mutations and that the gains target squamous cell carcinomas. As these alterations confer a growth advantage to the cancer cells, targeting the PI3K/Akt pathway is a potential therapeutic option for squamous cell lung cancers. It is of particular interest to target squamous cell carcinomas, the commonest form of lung cancer in ever smokers (38), as most recent trials of targeted agents for lung cancer have focused on adenocarcinomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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