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A Gene-Alteration Profile of Human Lung Cancer Cell Lines

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ABSTRACT: Aberrant proteins encoded from genes altered in tumors drive cancer development and may also be therapeutic targets. Here we derived a comprehensive gene-alteration profile of lung cancer cell lines. We tested 17 genes in a panel of 88 lung cancer cell lines and found the rates of alteration to be higher than previously thought. Nearly all cells feature inactivation at *TP53* and *CDKN2A* or *RB1*, whereas *BRAF*, *MET*, *ERBB2*, and *NRAS* alterations were infrequent. A preferential accumulation of alterations among histopathological types and a mutually exclusive occurrence of alterations of *CDKN2A* and *RB1* as well as of *KRAS*, epidermal growth factor receptor (*EGFR*), *NRAS*, and *ERBB2* were seen. Moreover, in non-small-cell lung cancer (NSCLC), concomitant activation of signal transduction pathways known to converge in mammalian target of rapamycin (mTOR) was common. Cells with single activation of ERBB2, PTEN, or MET signaling showed greater sensitivity to cell-growth inhibition induced by erlotinib, LY294002, and PHA665752, respectively, than did cells featuring simultaneous activation of these pathways, underlining the need for combined therapeutic strategies in targeted cancer treatments. In conclusion, our gene-alteration landscape of lung cancer cell lines provides insights into how gene alterations accumulate and biological pathways interact in cancer.
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KEY WORDS: lung cancer; oncogenes; tumor suppressors; tyrosine kinase inhibitors

Introduction

Characterization of accumulated genetic alterations in cancer cells is important not only to understand tumor biology, but also to guide drug design and select patients who might benefit from a given targeted cancer therapy. The promise of using proteins encoded by mutated cancer genes, mainly kinases encoded by oncogenes, as molecular targets for the development of novel therapies, drives endeavors to identify novel mutated cancer genes and to create catalogues of somatic mutations in cancer [Wang et al., 2004; Sjoblom et al., 2006; Greenman et al., 2007; Thomas et al., 2007]. The paradigm of the latter is the Catalogue of Somatic Mutations in Cancer (COSMIC) database of the Wellcome Trust Sanger Institute (www.sanger.ac.uk/cosmic) [Forbes et al., 2006], which brings together data on the mutation status of hundreds of cancer-related genes in primary tumors and cancer cell lines from a wide variety of tumor types.

In the particular case of lung cancer, several gene alterations are known to contribute to its development, including activating mutations and gene amplification at the oncogenes *BRAF* (MIM# 164757), epidermal growth factor receptor (*EGFR*) (MIM# 131550), *ERBB2* (MIM# 164870), *KRAS* (MIM# 190070), *NRAS* (MIM# 164790), *PIK3CA* (MIM# 1171834), *MYC* (MIM# 190080), *MYCL1* (MIM# 164850), and *MYCN* (MIM# 164840), as well as inactivating intragenic mutations, homozygous deletions, and promoter hypermethylation at the tumor suppressor genes *BRG1/SMARCA4* (MIM# 603254), *LKB1/STK11* (MIM# 602216), *PTEN* (MIM# 601728), *CDKN2A* (MIM# 600160), *RB1* (MIM# 180200), and *TP53* (MIM# 191170) [Sanchez-Cespedes 2007; Medina et al., 2008]. Some of these gene alterations are known to be specific to lung tumor histologies [Westra et al., 1993; Otterson et al., 1994; Kelley et al., 1995; Sanchez-Cespedes, 2007; Medina et al., 2008]. In addition, it is also well established that some gene alterations are mutually exclusive, as is the case for pairs of genes, such as *KRAS* and *EGFR*, or *CDKN2A* and *RB1* [Otterson et al., 1994; Lynch et al., 2004; Paez et al., 2004], that encode proteins acting in the same signaling pathway. However, a profile of alterations at multiple well-known cancer genes in a large panel of lung cancers has never been reported. This limits our understanding of how gene alterations are distributed among lung tumors and how they interact with one another.

Here, we attempt to delineate the gene-alteration profile of lung cancer cell lines by screening for alterations of seventeen well-known cancer genes, including point mutations at *AKT1* (MIM# 164730) and *EML4-ALK* (MIM# 607442 for *EML4* and MIM# 105590 for *ALK*) fusions, a small inversion within chromosome 2p recently reported in a small subset of non-small-cell lung cancers (NSCLCs) [Carpten et al., 2007; Soda et al., 2007]. We examined the association between the genetic alteration profile and the response to specific small molecule inhibitors.

Additional Supporting Information may be found in the online version of this article.

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Material and Methods

Cell Lines

Cells were maintained in culture flasks in either DMEM (A549, NCI-H1299, NCI-H23, Calu-3, NCI-H522, and EBC1) or RPMI 1640 (NCI-H446, NCI-H1650, NCI-H460, and NCI-N417) (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 50 mg/ml penicillin/streptomycin, and 2.5 μg/ml fungizone. Cultures were kept at 37°C in a humidified atmosphere of 5% CO_2/95% air. DNA, RNA, and protein were extracted using standard protocols.

Screening for Gene Mutations and Deletions

Screening for mutations in *AKT1* (exon 3), *BRAF* (exons 11 and 15), *MET* (MIM♯ 164860) (exons 16–20), *ERBB2* (exon 20), *EGFR* (exons 18–21), *NRAS* (codons 12, 13, and 61), *PIK3CA* (exons 1, 9, and 20), *PTEN* (exons 2–9), and *CDKN2A* (exons 1–3) was performed by directly sequencing PCR products using primers and conditions that have been previously described [Matsumoto et al., 2007; Angulo et al., 2008; Medina et al., 2008], or that are available upon request. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG transition initiation codon in the reference sequence. We considered the presence of homozygous deletions when there was a reproducible absence of PCR product of one or more consecutive exons. The mutational status of *STK11*, *SMARCA4*, *KRAS*, and *TP53* was either determined for those cases with incomplete/conflicting information or gathered from previous publications [Harbors et al., 1988; Yokota et al., 1988; Otterson et al., 1994; Shimizu et al., 1994; Matsumoto et al., 2007; Angulo et al., 2008] (Supp. Table S1) or from the Wellcome Trust Sanger Institute's Cancer Cell Line Project website (www.sanger.ac.uk/cosmic). In those cases where mutation/deletion data were not available, cells with a reported absence of RB protein expression were classified as *RB1*-mutant. The presence of the *EML4-ALK* fusion gene was tested according to previously published conditions [Soda et al., 2007].

Promoter Hypermethylation

The determination of promoter hypermethylation at *CDKN2A* was evaluated by bisulfite treatment of the genomic DNA and subsequent methylation-specific PCR, using previously published protocols [Esteller et al., 2001].

Real-Time Quantitative Genomic PCR for Determining Gene Amplification

To determine *MET*, *ERBB2*, *MYC*, *MYCL*, and *MYCN* amplification we used quantitative real-time genomic PCR. The conditions and primers used for *MYC*, *MYCN* and *MYCL* have been previously described [Medina et al., 2008]. *ERBB2* and *MET* primers and PCR conditions are available upon request. The copy number of genomic DNA was measured by SYBR green using an ABI Prism 7900 Sequence Detector (Applied Biosystems, Foster City, CA).

Inhibitors and Viability Assay

Rapamycin (mammalian target of rapamycin [mTOR] inhibitor) and LY-294002 (PI3K inhibitor) were obtained from Calbiochem (La Jolla, CA) and PHA665752 (MET inhibitor) from Tocris Bioscience (Ellisville, MI). Erlotinib (N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)-4-quinazolinamine) (EGFR inhibitor) was a gift from Roche Pharmaceuticals (Mannheim, Germany). Erlotinib tablets were ground to powder and dissolved in pure dimethyl sulfoxide (DMSO) to the desired concentration. For the cell-survival assays, cells were seeded at a density of 5,000 cells/well (15,000 cells/well for N417) on 96-well plates. They were allowed to recover for 12 hr before adding the drugs. Cells were exposed to various concentrations of each drug for 48 or 72 hr, and then the viable cell number was measured by the 3-(4,5-dimethylthiazol-2)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 10 μl of a solution of 5 mg/ml MTT (Sigma Chemical, Zwijndrecht, The Netherlands) was added to each well. After incubation for 3 hr at 37°C, the medium was discarded, the formed formazan crystals were dissolved in 100 μl DMSO and absorbance was determined at 596 nm by means of a microplate reader (Bio-Rad, Hercules, CA). Viabilities were expressed as a percentage of the untreated controls. The 50% growth inhibition (IC_{50}) was determined from the dose-response curve. Results are presented as the median of at least two independent experiments performed in triplicate for each cell line and each compound.

Antibodies and Western Blot Analysis

Anti-phospho-AKT (S473), anti-AKT, anti-S6, anti-phospho-S6 (S235/236), anti-phospho-MET (Y1234/Y1235), and anti-MET were obtained from Cell Signaling Technology (Beverly, MA). For western blotting, cells were seeded in 12-well culture plates and, after incubating for 24 hr with the designated drug, were scraped from the dishes into lysis buffer. Forty micrograms (μg) of total protein were separated by SDS-PAGE, transferred to a PVDF membrane, and blotted with the appropriate antibody according to the manufacturer's instructions.

Results

Gene Alteration Profiles of a Lung Cancer Cell Line Panel

To accurately determine the frequency of point mutations and homozygous/intragenic deletions of known cancer genes in lung cancer, avoiding the masking effect of the admixture with nonmalignant cells, we chose to screen cancer cell lines, including small-cell lung cancer (SCLC), squamous cell carcinomas (SCC), adenocarcinomas (AC), large-cell carcinomas (LCC), and carcinoids. Eighty-eight lung cancer cell lines were tested for alterations at 17 genes: *AKT1*, *BRAF*, *MET*, *EGFR*, *ERBB2*, *KRAS*, *STK11*, *MYC*, *MYCL*, *MYCN*, *NRAS*, *PIK3CA*, *PTEN*, *CDKN2A*, *RB1*, and *TP53*, as well as the *EML4-ALK* fusion. Alterations were present in all genes except *AKT1*. The *EML4-ALK* fusion was never detected. A total of 98% (86/88) of the cell lines had alterations of at least at one of the genes tested (Supp. Table S1 and Supp. Fig. S1). As expected, alterations in tumor-suppressor genes were homozygous whereas they were often heterozygous in oncogenes. Although two different heterozygous *TP53* mutations were detected in three cell lines, these mutations are likely to have occurred in each of both alleles resulting in the complete and biallelic inactivation of the *TP53* gene. The frequency of alterations when considering all histological types, from the highest to the lowest, were ranked as follows: *TP53* (79%), *CDKN2A* (59%), *RB1* (35%), *STK11* (27%), *MYC*-family (20%), *KRAS* (17%), *PTEN* (11%), *PIK3CA* (8%), *EGFR* (7%), *NRAS* (6%), *MET* (5%), *BRAF* (2%), and *ERBB2* (2%). The present study does not extend to mutation analysis at another key tumor-suppressor gene, *SMARCA4*, which has recently been found to be frequently altered in NSCLC [Medina

et al., 2008]. Data on the mutation status of *SMARCA4* for some cell lines is also provided in Supp. Table S1.

To determine possible cell culture artifacts we compared the mutational profile of lung cancer cell lines and lung primary tumors. The mutational status of the *TP53*, *STK11*, *KRAS*, *PIK3CA*, *EGFR*, and *BRAF* genes was available for non-small-cell lung primary tumors [Angulo et al., 2008]. The ranking of the most commonly mutated genes in lung primary tumors (*TP53* > *KRAS* > *STK11* > *EGFR* > *PIK3CA* > *BRAF*) was very similar to that in cell lines. However, the frequency of mutations at any gene in primary tumors was about half that in lung cancer cell lines (Supp. Fig. S2), suggesting a reduced effectiveness in the detection of gene alterations in primary tumors, probably due to contamination by normal cells. Alternatively, it is also possible that primary tumors are more heterogeneous than cell lines with respect to the accumulated genetic alterations. Since there are models for stepwise accumulation of genetic alterations both for lung AC and SCC, we can not completely discard that these differences arise as a consequence of different progression stages between the tumors and cell lines analyzed.

Gene Alterations and Histopathological Correlations

The distribution of gene alterations among patient characteristics and tumor histopathologies are summarized in Table 1. As previously described, alterations in *CDKN2A* and *STK11* were preferentially found in NSCLC, whereas alterations in *PTEN*, *RB1*, and in the *MYC* family of genes, especially *MYCL* and *MYCN*, were more common in SCLC. It is also interesting to note that mutations at other components of the *EGFR/KRAS* signal transduction pathway, i.e., *EGFR*, *ERBB2*, *BRAF*, and *NRAS*, predominate in lung AC. The differences did not reach statistical significance probably due to the few number of cell lines with mutations at those genes. However, when combined together, mutations at any of the different components of the *KRAS* pathway (*EGFR*, *ERBB2*, *KRAS*, *NRAS*, and *BRAF*) were significantly more frequent in lung AC as compared to SCCs ($P < 0.05$; Fisher's exact test) and in NSCLC as compared to SCLC ($P < 0.00005$; Fisher's exact test). Alterations at *TP53* were present in a similar frequency in both SCLC and NSCLC, indicating that its inactivation is required for the development of all histopathological types of lung cancer. Although very low frequency, mutations at *PIK3CA* were also found in NSCLC and SCLC. The mutations found in the later correspond to novel variants that need verification.

As previously reported, mutations at *KRAS* and *EGFR* predominate in tumors from Caucasian and Asian patients, respectively. However, a new observation that arises from our study is the accumulation of alterations at the *MYC*-family of gene in tumors from patients of Caucasian origin ($P < 0.05$; Fisher's exact test). No associations were detected between alterations at any gene and gender, or age, nor were gene alterations seen to have accumulated in tumors of older patients. Rather than a definitive observation, the lack of association between the presence of mutations at *EGFR* and *KRAS* with tumors from nonsmokers and smokers, respectively, is likely due to the lack of information on the smoking habit of many the individuals.

Identification of Novel Variants

In addition to well-known somatic mutations with an oncogenic effect within the helical and kinase domains of *PIK3CA* [Samuels et al., 2004; Gymnopoulos et al., 2007; Angulo et al., 2008], we identified two novel variants, both located near well-characterized mutation hotspots. One of these is an insertion of 387 nt after the termination codon TGA that results in the duplication of amino acids 1,051 to 1,068 (Fig. 1B) and the other is a p.D1029Y substitution. Since no matched normal DNA was available for these cell lines, we could not test whether these mutations are germline polymorphisms or tumor-specific mutations. Four cell lines carried *MET* alterations, including gene amplification and two novel variants, p.L1158F (in the HCC15 cells) and p.T1259K (in the H1963 cells) (Fig. 1B and C). Again, due to the lack of normal matched DNA for these cell lines we could not verify the somatic nature of the amino acid substitutions. However, the absence of constitutive MET activation indicated by the lack of $pMET^{Y1234/Y1235}$ in these cell lines strongly argues against an oncogenic role for the variants (Fig. 1D). The H441, Calu3, HCC366, and HCC78 cells that were reported to have high levels of $pMET^{Y1234/Y1235}$ [Rikova et al., 2007] did not feature gene amplification or point mutations within the hotspots tested here.

Cooperation of Several Biological Pathways in Lung Carcinogenesis

It is widely accepted that alterations of genes in the same biological pathways are not redundant in cancer cells. Accordingly, genes that are altered in a mutually exclusive manner are likely to encode proteins that act in the same biological pathway. This hypothesis has been extensively borne out in lung cancer cells by the lack of concomitant alterations at *RB1* and *CDKN2A*, and at *EGFR* and *KRAS*. Our data also confirm the mutually exclusive nature of these pairs of alterations (Fig. 1A). Likewise, alterations at *ERBB2* and *NRAS* did not occur in the same cell lines or in cells carrying *EGFR* and *KRAS* mutations, consistent with their participation in the same signal transduction pathway. *PTEN* and *PIK3CA*, which are both encoding proteins that modulate the intracellular levels of the phosphoinositide-3,4,5-trisphosphate (PIP3), were also found to be mutated in a mutually exclusive manner. Only one cell line, Lu134, with a homozygous deletion at *PTEN*, had a concomitant change at *PIK3CA*. The *PIK3CA* variant is a p.D1029Y substitution, which has not been described before and for which there is no evidence of its somatic nature. On the other hand, there were concomitant *BRAF*- and *NRAS*-activating mutations in the H2087 lung adenocarcinoma cells. The somatic nature of the p.L597V mutation in *BRAF* was confirmed after sequencing the DNA of the corresponding lymphoblastoid line (BL-H2087). On the other hand, simultaneous mutations in signal transduction pathways that are known to converge in the modulation of mTOR activity, such as *MET*, *PIK3CA/PTEN*, *STK11*, and *KRAS/EGFR/NRAS/ERBB2*, were present in some cell lines, implying cooperation in cancer development. Namely, 17 (28%) of the 61 NSCLC cell lines carried single mutations, whereas 16 (26%) and two (3%) of them carried double and triple mutations, respectively, in any of this group of genes.

Correlation of Acquired Genetic Alterations With Sensitivity to Small Molecule Inhibitors

To understand a possible effect of these genetic alterations on the primary resistance to tyrosine kinase inhibitors (TKIs) and other small molecule inhibitors, we selected a panel of 10 lung cancer cell lines with a known genetic background for *KRAS*, *STK11*, *EGFR*, *PTEN*, *PIK3CA*, and *MET*, and tested the sensitivity to treatment with inhibitors of PI3K (LY294002), mTOR (rapamycin), MET (PHA665752), and EGFR (erlotinib). As

Table 1. Distribution of the Indicated Mutations Among the Different Characteristics of the Lung Cancer Cell Lines

		TP53		*CDKN2A*		*RB1*		*STK11*		ANY (*MYC*, *MYCL*, *MYCN*)		*KRAS*[a]		*PTEN*		*PIK3CA*		*EGFR*[b]		*NRAS*[b]		*MET*		*BRAF*[b]		*ERBB2*[b]		ANY (*KRAS*, *EGFR*, *NRAS*, *BRAF*, *ERBB2*)	
	Total	WT	MUT	WT	MUT	WT	MUT	WT	MUT	WT	MUT	WT	MUT	WT	MUT	WT	MUT	WT	MUT	WT	MUT	WT	MUT	WT	MUT	WT	MUT	WT	MUT
Total		17	70	36	52	47	26	63	24	70	18	72	15	78	10	81	7	82	6	83	5	81	4	86	2	86	2	57	29
		80%		59%		36%		31%		20%		17%		11%		8%		7%		6%		5%		2%		2%		33%	
Age (years)																													
≤40	10	1	9	3	7	5	1	7	3	10	0	6	3	8	2	10	0	5	2	7	0	9	1	7	0	6	1	4	6
40–60	38	10	28	17	21	24	12	27	11	27	11	14	5	35	3	37	1	15	2	16	1	36	2	15	1	16	1	24	13
≥60	30	3	26	15	15	12	10	23	6	25	5	10	3	25	5	27	3	9	0	8	2	29	1	10	1	10	0	24	6
Gender																													
Female	17	5	12	7	10	8	4	11	6	13	4	5	4	15	2	16	1	9	1	9	1	16	0	9	1	9	1	9	7
Male	66	7	58	28	38	38	19	49	16	52	14	27	9	58	8	60	6	23	4	25	2	61	3	26	1	26	1	48	18
Origin																													
Asian	32	5	26	12	20	19	6	24	8	30	2	17	2	29	3	29	3	10	5	14	2	31	1	15	0	15	0	23	8
Black	5	0	5	3	2	2	3	4	1	4	1	1	1	5	0	5	0	2	0	2	0	3	1	2	0	2	0	4	1
Caucasian	38	9	29	19	19	22	14	28	9	27	11	12	7	31	7	36	2	15	1	14	2	36	0	14	2	14	2	24	14
										$P<0.05$*		$P<0.06$*						$P<0.07$*											
Smoker																													
Yes	34	7	27	21	13	16	16	26	7	25	9	10	4	27	7	33	1	11	1	11	1	30	2	10	2	11	1	23	11
No	9	1	8	2	7	7	2	8	1	8	1	4	2	8	1	8	1	4	0	3	1	8	0	4	0	4	0	6	3
HIST																													
AC	39	6	33	8	31	31	1	24	15	34	5	30	9	38	1	37	2	33	6	36	3	38	1	37	2	37	2	18	21
SCC	13	2	11	3	10	6	1	9	4	12	1	12	1	11	2	12	1	13	0	12	1	11	0	13	0	13	0	11	2
														$P=0.15$														$P<0.05$	
LCC	9	2	7	2	7	7	1	5	4	7	2	5	4	8	1	7	2	9	0	8	1	9	0	9	0	9	0	4	5
SCLC	24	6	17	22	2	2	19	23	0	15	9	24	0	19	5	22	2	24	0	24	0	23	1	24	0	24	0	23	0
Others[c]	3	1	2	1	2	2	1	2	1	2	1	2	1	2	1	3	0	3	0	3	0	1	0	3	0	3	0	2	1
HIST																													
NSCLC	61	9	52	13	48	44	3	38	23	53	8	47	13	57	4	56	5	55	6	56	5	58	3	59	2	59	2	35	29
SCLC	24	6	17	22	2	2	19	23	0	15	9	23	0	19	5	22	2	24	0	24	0	23	1	24	0	24	0	23	0
				$P<0.0001$		$P<0.0001$		$P<0.0001$		$P<0.05$		$P<0.05$		$P<0.05$														$P<0.00005$	

[a]Analysis performed only for the adenocarcinoma and large-cell carcinoma cell lines.
[b]Analysis performed only for the adenocarcinoma cell lines.
[c]Includes the following categories: one mesothelioma, one carcinoid, and one neuroendocrine.
*Asian vs. Caucasian comparison.
HIST, histopathological type.

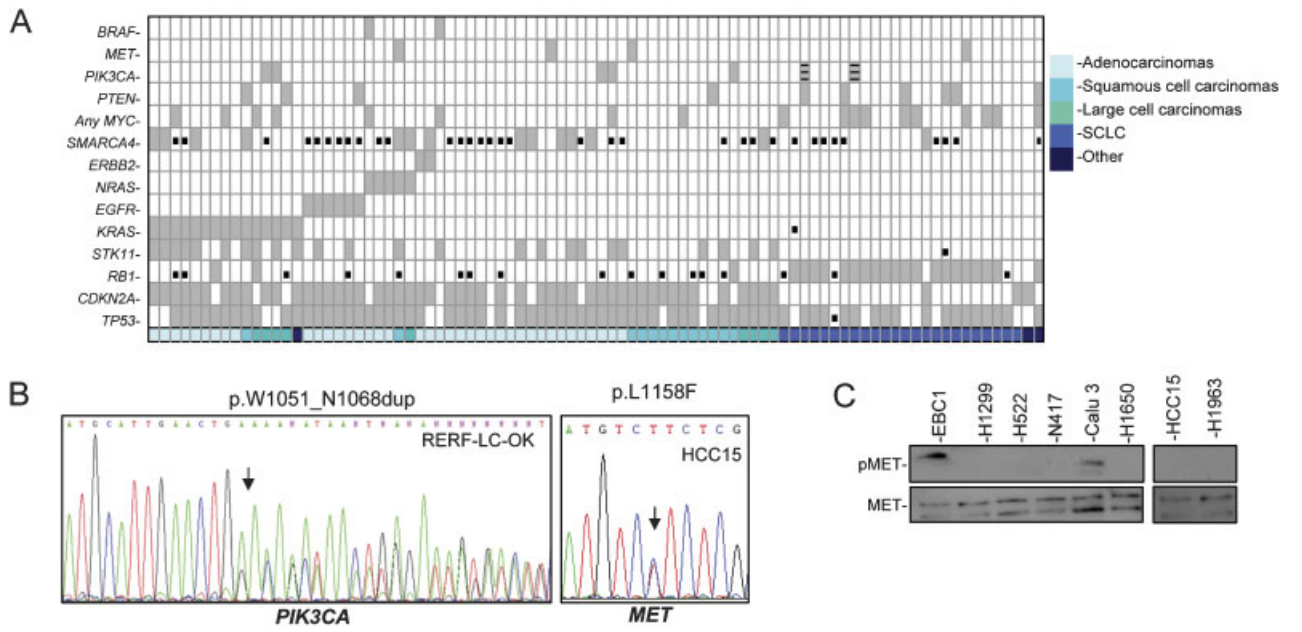

Figure 1. Gene alterations in lung cancer cell lines. **A:** Profile of genes altered in human lung cancer cell lines. The presence of alterations is indicated by gray bars. Black squares indicate no data. The black lines in the *PIK3CA* oncogene refer to the two variants of unknown oncogenic potential. The histopathology is also shown. **B:** *PIK3CA* and *MET* variants in the RERF-LC-OK and HCC15 cell lines. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG transition initiation codon in the reference sequence **C:** *MET* gene amplification in lung cancer cell lines revealed by quantitative PCR. The relative *MET* copy number was determined by comparison with an unrelated control locus, MDH2, on chromosome 7q11. Cells with *MET* amplification are indicated with an arrow. **D:** Western blot anti-phospho-MET ($pMET^{Y1234/Y1235}$) and anti-MET (MET) in the indicated cell lines. Constitutive MET activation is present in the EBC-1 and Calu-3 cells, but not in the HCC15 and H1963 cells, which carry gene variants of unknown biological significance.

subrogate markers to test the ability of the drug to inhibit its target molecule we measured the levels of $pAKT^{Ser473}$ (for PI3K and EGFR inhibitors), $pS6^{Ser235/236}$ (for mTOR inhibitor), and $pMET^{Y1234/Y1235}$ (for MET inhibitor). The calculated IC_{50} for the different compounds is summarized in Figure 2A. A marked genotype–drug sensitivity association was observed for the Calu-3 and EBC-1 cells, which were highly responsive to growth inhibition triggered by erlotinib and PHA665752 compounds, respectively. The effectiveness of these treatments was also measured by their ability to decrease phosphorylation at their target molecules or at downstream effectors (Fig. 2B). We did not observe a low IC_{50} in response to treatment with PHA665752, in the H1963 or HCC15 cell lines (data not shown). These carry amino acid substitutions at the tyrosine kinase domain of *MET*, which is further indication that these variants are not functionally significant. Similarly, the Calu-3 cells that carry high levels of MET phosphorylation (Fig. 1D) but do not exhibit gene amplification or mutations were insensitive to PHA665752. Interestingly, the H522 cells evidenced a strong sensitivity to PHA665752. These cells neither carry amplification/point mutations at *MET* nor MET phosphorylation. Thus, the characterization of the gene alterations underlying the sensitivity of these cells to MET inhibitors will be of interest. Although the differences were not as marked, we also noted that sensitivity to LY294002, as indicated by the lower IC_{50}, was increased in the H446 and N417 cell lines, both of which are *PTEN*-deficient. Similarly, the lowest IC_{50} to rapamycin was observed for the N417, H446, EBC-1, and Calu-3 cells (Fig. 2A and B). Some of these cells carry constitutive activation of AKT due to the presence of *PTEN* inactivation (the N417 and H446), or to *ERBB2* gene amplification (Calu-3). Intriguingly, the triple mutant *KRAS-STK11-PIK3CA* (H460) and *EGFR-PTEN* (H1650) cells were extremely resistant to rapamycin, LY294002, and erlotinib. Thus, we investigated the effect of the combined treatment with erlotinib and LY294002 on cell growth, and found that the addition of erlotinib significantly increased the efficiency of cell-growth inhibition of the LY294002 compound in H1650 cells, but not in H460 cells (Fig. 3A and B).

Discussion

We provide a detailed gene-alteration profile of lung cancer cells of distinct histologies. In full compliance with Knudson's two-hit hypothesis [Knudson, 1971], mutations in tumor suppressors, but not in oncogenes, were always homozygous. We also confirmed the disproportionately high frequency of occurrence of some gene alterations in specific histological types, which probably reflects differences in the cell type of origin. The overall profile of genes mutated in lung cancer was comparable between lung primary tumors and lung cancer cell lines. However, the frequency of mutations at any gene was higher in cell lines, which strongly implies a masking effect due to the admixture of nonmalignant cells that hinders the detection of point mutations and insertions/deletions in the primary tumors. This obstacle has been noted before [Sanchez-Cespedes, 2007; Thomas et al., 2006] and is a significant problem that may be solved by the use of a novel generation of sequencers [Thomas et al., 2006], or by other technical approaches like careful microdissection of tumor cells.

TP53 was the most frequently altered gene in the lung cancer cell lines. Nearly 80% of the cell lines carry alterations of this tumor suppressor. Similarly, alterations at the cell cycle components, either *RB* or *CDKN2A*, were also extremely common. The high frequency of *TP53* and *CDKN2A/RB1* alterations in all histopathologies is a demonstration of their important role in lung cancer development. It is tempting to speculate that *TP53* and *CDKN2A/RB1* inactivation in lung cancer may be universal and are thus a requisite for the evolution of lung tumors.

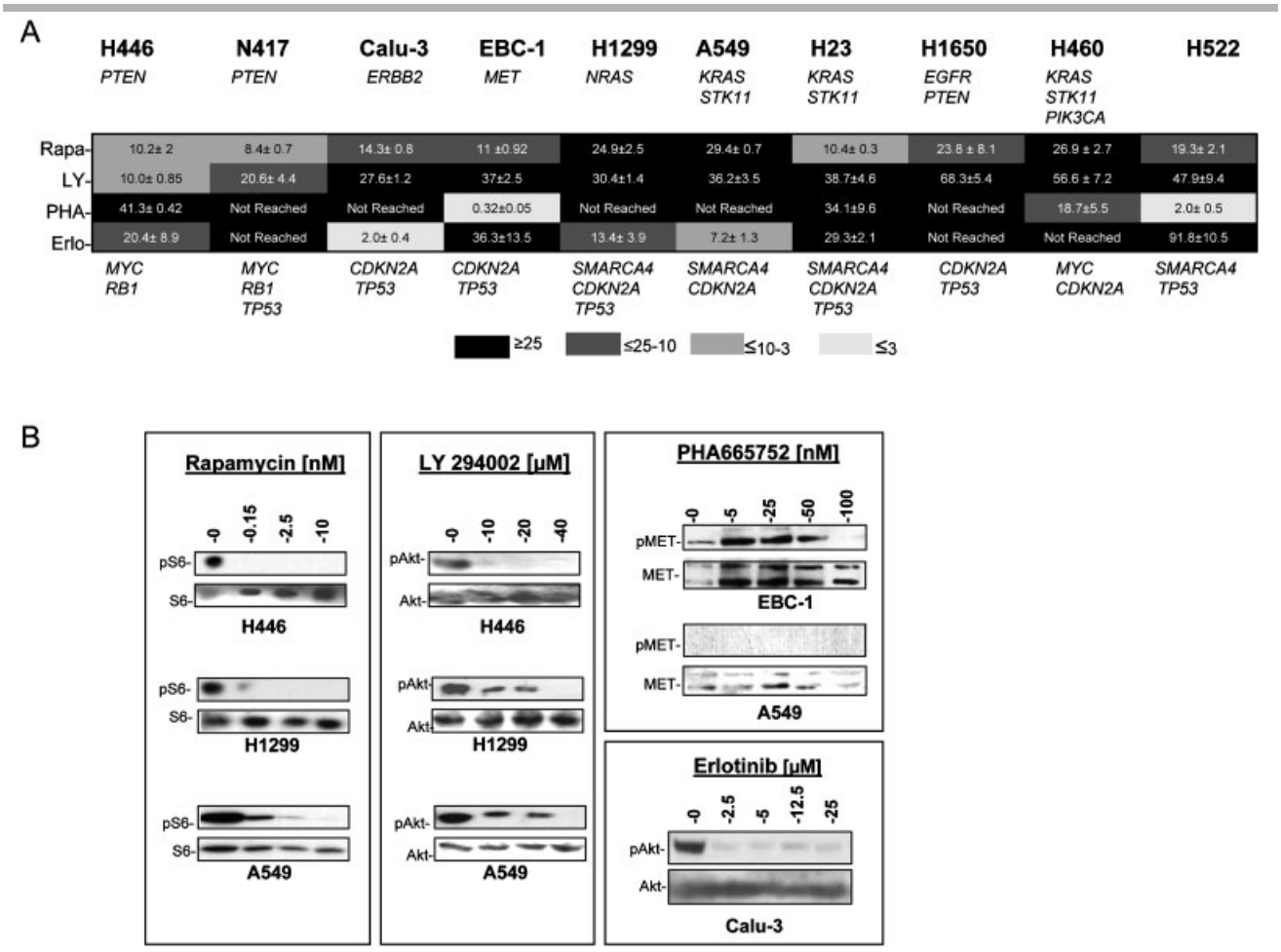

Figure 2. Genotype of the cell lines and sensitivity to specific inhibitors. **A:** The IC_{50} (μM) for each compound (RAPA, rapamycin; LY, LY294002, PHA, PHA665752; and Erlo, erlotinib) is indicated within the boxes. Treatments were applied for 72 hr. **B:** Immunoblotting analysis depicting the decreased phosphorylation of the indicated protein upon administering increasing concentrations of the compound. Treatments were applied for 24 hr.

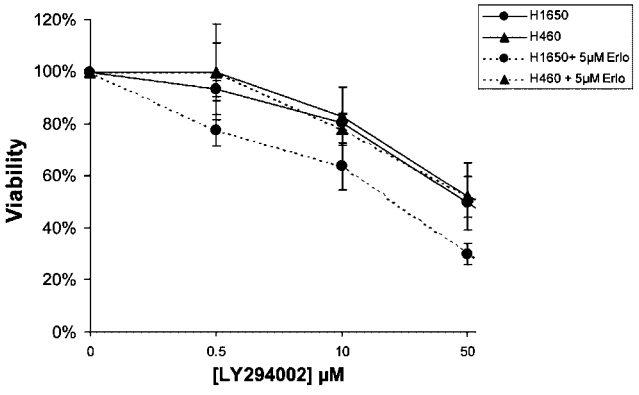

Figure 3. Cell-growth inhibition upon administering combined LY294002 and erlotinib treatment. Lines represent the cell survival relative to untreated controls of the MTT assays in the H1650 and H460 cells treated with increasing concentrations of LY294002, alone or with 5 μM erlotinib for 72 hr. Error bars indicate the standard deviation of three replicates.

Conversely, alterations at some oncogenes, such as *BRAF*, *ERBB2*, and *MET*, were infrequent.

It was remarkable the differences in the activation of components of the *KRAS* pathway among the lung cancer histopathologies. While alterations at any of the *BRAF*, *EGFR*, *ERBB2*, *KRAS*, or *NRAS* was significantly more common in AC as compared to SCC, virtually none of the SCLC carry alterations at any of those genes. This strongly points out towards completely different mechanisms of carcinogenesis for NSCLC and SCLC and likely accounts for the distinct clinical behavior of both types of lung cancer.

Although mutations outside the hotspots may increase the frequency of alterations at these genes to some extent, it seems certain that their contribution will be confined to a small subset of lung tumors. However, given that the encoded proteins are targets for small molecule inhibitors, the context in which these mutations arise (e.g., histological type, concomitant mutations at other genes) needs to be better understood. We confirmed the lack of concomitant mutations in those genes encoding proteins acting in the same biological pathway, such as *CDKN2A/RB1*, *KRAS/EGFR/ERBB2*, and *PIK3CA/PTEN*. Apart from these, simultaneous alterations were found in most of the other genes. Intriguingly, we also found that *BRAF-NRAS*, were genetically altered in the same cells, suggesting that the collaboration of the encoded proteins affects the development of the cancer. Similarly, it was previously reported that *BRAF* mutations involving codons other than 600 or 601 were highly likely to co-occur with a RAS family mutation [Thomas et al., 2007]. It is interesting to note the frequent concomitant activation of signal transduction pathways

that converge in the modulation of mTOR activity upon different stimuli, such as *KRAS/EGFR/ERBB2*, *PIK3CA/PTEN*, and *STK11* [Corradetti and Guan, 2006].

Selective small inhibitors against molecules that participate in different signaling pathways have been approved or are at various stages of development for clinical use in cancer patients. In this new scenario of targeted therapies, the response to a given therapeutic drug is likely to depend on the genetic background of the tumor. Similarly to previous observations [McDermott et al., 2007], our present results show how lung cancer cells with single alterations at *MET*, *PTEN*, or *ERBB2/EGFR* are sensitive to MET (PHA665752), PI3K (LY294002), and EGFR (erlotinib) inhibitors, respectively. However, this does not hold true in cells with activation of multiple signaling pathways, suggesting that there are interconnections among pathways that enable cells to bypass the negative effects on cell growth triggered by the small inhibitor. We found that in the originally resistant *EGFR/PTEN* double-mutant cells, erlotinib sensitized the cells to the effect of the LY294002 compound, which suggests that the use of drug combination strategies could improve sensitivity to specific therapies. Current efforts to understand the mechanisms of tumor resistance, especially to TKIs in lung cancer, further support this hypothesis [Rikova et al., 2007; Engelman et al., 2007]. Guo et al. [2008] reported that in *EGFR*-mutant cells which are sensitive to EGFR inhibitors, EGFR drives other receptors tyrosine kinases (RTKs) and a network of downstream signaling that collapse with drug treatment. In these cells, secondary drug resistance appears through the generation of novel gene alterations at another RTK, MET, preventing such collapse and thus bypassing the inhibitory effect of the drug. Taken together these observations are strong evidence that different signal transduction pathways assemble in networks, through the use of some common components. Beyond the contribution to the understanding of cell biology, our observations draw attention to the need to stratify tumors according to their genotype and histology and suggest that the combination of pathway-selective therapies will eventually be required for the treatment of many solid tumors.

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