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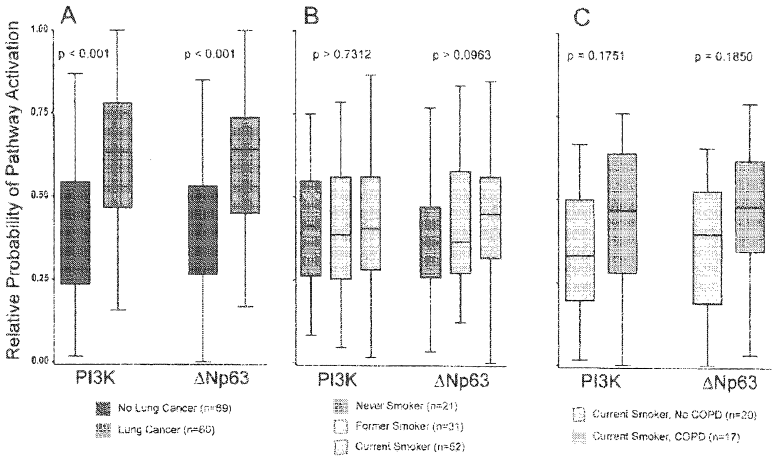

Fig. 1A-1C

(57) Abstract: The invention provides the identification of oncogenic pathways activated in cytologically normal airway cells of individuals having or at risk of having lung disease, as well as specific gene expression patterns (biomarkers) associated with pathway activation. These biomarkers and pathways may provide prognostic and/or diagnostic indicators in lung disease, e.g., lung cancer. Additionally, these pathways and biomarkers may provide therapeutic targets for the treatment of lung disease, as well as markers for the assessment of treatment efficacy.

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IDENTIFICATION OF NOVEL PATHWAYS FOR DRUG DEVELOPMENT FOR LUNG DISEASE

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. provisional application Serial No. 60/994,643, filed September 19, 2007, the entire teachings of which are incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

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BACKGROUND OF THE INVENTION

Cigarette smoke is the dominant cause of lung cancer in the United States, accounting for an estimated 90% of all cases [1]. However, the damage caused by cigarette smoke is not limited solely to the lung, but rather constitutes a 'field of injury' throughout the entire respiratory tract [2-6]. An important product of the field of injury hypothesis is the ability to glean clinically relevant information from cells collected in regions of the respiratory tract, such as the bronchial airway, that can be obtained in a less invasive manner than is typical of collecting primary lung tissue. Based on this approach, a gene expression-based biomarker measured in the

cytologically normal bronchial airway epithelium that can distinguish smokers with and without lung cancer has been developed [7]. This airway gene expression biomarker achieved 83% accuracy in predicting whether a smoker had a lung tumor in a prospective test set, and 94% accuracy when combined synergistically with clinical variables [7, 8].

Beyond serving as an early diagnostic tool for lung cancer, gene expression changes in the cytologically normal airway epithelium have the potential to improve our understanding of the signaling events deregulated during early stages of lung cancer. Lung cancer development in humans is a complex process involving multiple aberrant events that, when accumulated, lead to deregulation of crucial cell functions, including cell survival and proliferation. In primary tumors resected from patients with lung cancer, many signaling pathways have previously been found to be deregulated, such as p53, RAS and phosphatidylinositol 3-kinase (PI3K) [9-11]. Additionally, studies in the field of injury and field cancerization have found that some of the molecular changes presumed to be early in tumorigenesis are also reflected in histologically normal cells both neighboring and more distal to the primary tumor. For example, the same p53 mutation or loss of heterozygosity at a specific chromosomal region have been identified throughout the entire respiratory tract [5, 12].

Insight into the deregulation of oncogenic pathways in cytologically normal bronchial airway cells from smokers with lung cancer will help elucidate mechanisms involved in the progression into malignancy. Furthermore, understanding which pathways are deregulated could lead to therapeutic and chemoprophylactic opportunities at the pre-malignant stage of lung cancer.

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Any discussion of the prior art throughout the specification should in no way be considered as an admission that such prior art is widely known or forms part of common general knowledge in the field.

SUMMARY OF THE INVENTION

According to a first aspect, the present invention provides a method of identifying an individual at increased risk of lung disease, comprising determining the activation status of the PI3K pathway in a cytologically normal airway epithelial cell from said individual, wherein activation of the PI3K pathway is indicative that said individual is at increased risk of lung disease as compared with an individual in whom the PI3K pathway is not activated.

According to a second aspect, the present invention provides a method of identifying an individual at increased risk of lung disease, comprising determining the activation status of PKC in a cytologically normal airway epithelial cell from said individual, wherein activation of PKC is indicative that said individual is at increased risk of lung disease as compared with an individual in whom PKC is not activated.

According to a third aspect, the present invention provides a method of identifying an individual at increased risk of lung disease, comprising determining the activation status of IGF1R in a cytologically normal airway epithelial cell from said individual, wherein activation of IGF1R is indicative that said individual is at increased risk of lung disease as compared with an individual in whom IGF1R is not activated.

Unless the context clearly requires otherwise, throughout the description and the claims, the words “comprise”, “comprising”, and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of “including, but not limited to”.

It has been previously demonstrated that gene expression profiling of cytologically normal bronchial airway epithelium reflects a field of injury in smokers that can serve as a sensitive and specific diagnostic biomarker for lung cancer. Using gene expression signatures defined by *in vitro* perturbation of specific oncogenic pathways, as described herein a significant increase of phosphatidylinositol 3-kinase (PI3K) pathway activity was identified in the cytologically normal airway of smokers with lung cancer (n=129), as well as in lung tumor tissue (n=107). To evaluate whether increased activity of PI3K occurs prior to the development of lung cancer, cytologically normal airway epithelia in high-risk smokers with moderate-severe dysplastic lesions in their airway (n=14) were profiled, and higher levels of PI3K pathway gene expression were found as compared to the airway epithelium from healthy smokers without dysplasia

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(n=11). Further, PI3K activity was decreased in the airway of high-risk smokers who had significant regression of dysplasia following treatment with the chemoprophylactic agent myo-inositol (n=10). *In vitro* dilution experiments confirmed that myo-inositol inhibits the PI3K pathway, which not only proposes a mechanism of action for myo-inositol, but also reflects a potential therapeutic relationship between PI3K pathway activity and regression of airway dysplasia. Together, these findings suggest that deregulation of the PI3K pathway is an early, measurable and reversible step in the development of lung cancer, and that airway gene expression profiling in smokers may enable personalized approaches to chemoprophylaxis and therapy.

In one embodiment the invention provides biomarkers for oncogenic pathways activated in cytologically normal airway epithelial cells of individuals with lung disease. These biomarkers and pathways may provide prognostic and/or diagnostic indicators of lung disease, e.g., lung cancer. Additionally, these pathways and biomarkers may provide therapeutic targets for the treatment of lung disease, as well as markers for the assessment of treatment efficacy.

In another aspect the invention relates to the use of gene expression profiling methods to identify gene expression signatures of oncogenic pathways activated in lung disease, e.g., lung cancer. These gene expression signatures may provide prognostic or diagnostic indicators for lung disease, e.g., lung cancer. Moreover, oncogenic pathways which are activated in lung disease may be targets for therapeutic intervention. In one embodiment the oncogenic pathway can be, for example, one or more of the PI3K and Np63 pathways.

The invention also relates to a method of identifying an individual at increased risk of lung disease, comprising determining the activation status of the Np63 and/or PI3K pathway in a cytologically normal airway epithelial cell from said individual, wherein activation of the Np63 and/or PI3K pathway is indicative that said individual is at increased risk of lung disease as compared with an individual in whom the Np63 and/or PI3K pathway is not activated. In particular embodiments the individual is a smoker or a non-smoker. In other embodiments the lung disease is lung cancer. In one embodiment the activation status of the PI3K pathway is determined using gene expression data for one or more biomarkers of the PI3K pathway. For example, in some embodiments at least one of said one or more biomarkers is a gene which is increased upon PI3K activation, and in other embodiments at least one of said one or more biomarkers is a

gene which is decreased upon PI3K activation. Combinations of biomarkers which are increased and decreased upon PI3K activation may also be used. In particular embodiments at least one of said one or more biomarkers is a gene which is upstream of PI3K activation, while in other embodiments at least one of said one or more biomarkers is a gene which is downstream of PI3K activation.

In particular embodiments, expression data for said one or more biomarkers of the PI3K pathway is obtained using an oligonucleotide microarray. In other embodiments the activation status of the PI3K pathway is determined using one or more gene expression products of one or more biomarkers of the PI3K pathway. Said gene expression products may be nucleotide or amino acid products and can be detected using methods known in the art.

In some embodiments of the invention the activation status of the PI3K pathway is determined by assessing the activation of IGF1R, wherein activation of IGF1R is indicative of activation of the PI3K pathway. In other embodiments of the invention the activation status of the PI3K pathway is determined by assessing the activation of PKC, wherein activation of PKC is indicative of activation of the PI3K pathway.

The invention also relates to a method of identifying an individual at increased risk of lung disease, comprising determining the activation status of PKC in a cytologically normal airway epithelial cell from said individual, wherein activation of PKC is indicative that said individual is at increased risk of lung disease as compared with an individual in whom PKC is not activated.

The invention further relates to a method of identifying an individual at increased risk of lung disease, comprising determining the activation status of IGF1R in a

cytologically normal airway epithelial cell from said individual, wherein activation of IGF1R is indicative that said individual is at increased risk of lung disease as compared with an individual in whom IGF1R is not activated.

In other embodiments the invention provides an oligonucleotide array having immobilized thereon one or more probes for one or more biomarkers of the PI3K pathway, and wherein said array does not have immobilized thereon probes for other biomarkers. In preferred embodiments said one or more biomarkers of the PI3K pathway are selected from the group consisting of IGF1R, PKC, the biomarkers disclosed in [29], and combinations thereof.

The invention also relates to a method of reducing the risk of lung disease in an individual comprising administering to an individual at risk of lung disease one or more agents (e.g., one or more agents, regimens or treatments or combinations thereof) which inhibit the PI3K pathway. In particular embodiments the PI3K pathway is activated in said individual prior to administration of said one or more agents. In one embodiment the lung disease is lung cancer. In another embodiment said one or more agents are administered to said individual prophylactically before the development of lung disease.

In another embodiment the invention relates to a method of differentially classifying a cytologically normal test airway epithelial cell, comprising identifying a gene expression signature associated with activation of a biological pathway of interest in a normal airway epithelial cell; assessing gene expression in differentially classified airway epithelial cells to identify one or more correlations between classification of an airway epithelial cell and activation of a biological pathway of interest; and assessing gene expression in a cytologically normal test

airway epithelial cell, wherein the gene expression profile of the cytologically normal airway epithelial cell to be classified indicates whether the biological pathway of interest is activated and thus differentially classifies the cell.

In particular embodiments the biological pathway of interest is an oncogenic pathway. In some embodiments the differential classification is increased risk of disease versus decreased risk of disease, while in other embodiments the differential classification is response to treatment versus non-response to treatment.

In one embodiment, the invention provides a method for identifying the activation of an oncogenic pathway in a mammal having or at risk of having lung disease, e.g., lung cancer. The method may include: (a) providing a biological sample, e.g., a biological sample from an airway passage of the mammal, wherein the biological sample comprises a gene expression product (e.g., mRNA or protein) from at least one gene that is indicative of activation of said pathway, and (b) detecting the expression of said gene. For example, the pathway can be one or more of the following: Ras, Myc, E2F3, beta-catenin, Src, Np63, PI3K and combinations thereof. The mammal can be, for example, a human. Biological samples may be provided, for example, from bronchial, nasal or buccal epithelium, or from biopsied tissue samples. In one embodiment, detection of gene expression is accomplished using an oligonucleotide array having immobilized thereon one or more nucleotide sequences or fragments thereof which are probes for the relevant gene(s). Identification of activation of an oncogenic pathway may indicate that the mammal is a candidate for treatment to inhibit activation of said pathway or for additional or more frequent screening to identify development of disease, e.g., cancer.

In another embodiment, the invention provides a method of screening candidate therapeutic agents which may be useful in the treatment of lung disease, e.g., lung cancer. For example, candidate agents may be screened for their ability to modulate (e.g., inhibit) the activation of an oncogenic pathway identified by methods described herein as associated with lung disease. The agent's ability to modulate activation of the pathway may be assessed, for example, by its ability to alter a gene expression signature of an oncogenic pathway from a signature which is associated with disease to a signature which is not associated with disease, e.g., is normal. Alternatively the candidate therapeutic agent may be assessed for its ability to modulate a specific functional effect or readout of the pathway. Agents identified as having the ability to inhibit an activated oncogenic pathway associated with lung disease may be suitable for treatment of lung disease in a mammal.

In addition, the efficacy of a treatment regimen can be evaluated by assessing the gene expression signature of a mammal at various time points over the course of treatment. A shift in the gene expression signature of an oncogenic pathway from one associated with disease to one not associated with disease, e.g., to a normal signature, is indicative of efficacious treatment. Similarly absence of a shift in the gene expression signature toward a normal signature is indicative that treatment is not efficacious and that perhaps alternative treatment regimens are indicated.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A-1C show that PI3K and ΔNp63 are differentially activated in smokers with lung cancer.

Using binary regression models trained on in vitro gene expression signatures, pathway activation probabilities were calculated in samples obtained from the cytologically normal airway. Pathway levels are summarized using box plots, where the bar represents the median value, the box denotes the range of the data points from the 25th to 75th percentile, and the whiskers specify the range of the remaining 1st and 4th quartile. As shown in Fig. 1A, when grouping the activation levels by lung cancer status (blue for no lung cancer, red for lung cancer), two pathways were found to be statistically different after random permutation tests: PI3K ($p<0.001$), and ΔNp63 ($p<0.001$). To account for variables that could possibly confound the observed differences in pathway activation seen in the airway of smokers with lung cancer, pathway activation probabilities were also calculated for healthy never (green), former (brown) and current smokers (gray) (Fig. 1B), as well as current smokers with (orange) or without (gray) chronic obstructive pulmonary disease (COPD) (Fig. 1C). Neither of the potential confounding variables showed a statistically significant difference in pathway activation.

Fig. 2 shows oncogenic pathway activity in lung tumor and adjacent normal tissue. Oncogenic pathway activity was calculated for a dataset of lung adenocarcinoma and adjacent normal tissue [32]. An increase in PI3K ($p<0.001$) and ΔNp63 ($p=0.002$) was observed when comparing adjacent normal and its paired tumor sample. Increases were also seen in Myc and E2F3, and there was a decrease in Src. Error whiskers are reported as SEM.

Figs. 3A-3B show the biochemical validation of PI3K activity in prospectively collected airway samples. Airway brushings were collected prospectively from patients under suspicion of having lung cancer in Boston and Utah. As shown

in Fig. 3A, kinase assays were used to measure in vivo levels of PI3K pathway activity. Patients with lung cancer generally had higher levels of PI3K activity than those without lung cancer. A subset of the Boston cohort had extra sample run on microarray so that computational predicted PI3K activity could be correlated to in vivo activity. The probability of pathway activity is shown below the patients that had extra samples. Pearson correlation of the computationally predicted PI3K activity and the biochemically measured activity was 0.48. As shown in Fig. 3B, Western blots querying proteins both upstream and downstream of PI3K were quantified and then correlated with PI3K kinase levels measured in Fig. 3A. Correlations are presented in a heatmap manner, where blue represents negative correlation, and red represents positive correlation. Correlation analysis is also broken down into all samples, only samples with lung cancer, and only control samples. Both p-IGF1R and p-PKC are positively correlated with PI3K activity in patients with lung cancer, suggesting possible sub-pathways driving the increased PI3K pathway activity in the airway of smokers with lung cancer.

Fig. 4 demonstrates that smokers with dysplasia have an increased activation of the PI3K pathway. Genes that increase when the PI3K pathway is activated, as defined by *in vitro* perturbation, are displayed in a heatmap. Blue represents low expression of a gene, while red represents higher expression of a gene. When comparing the expression levels of these genes in the cytologically normal bronchial airway of smokers with dysplasia against healthy smokers, an increased activation of the PI3K pathway in smokers with dysplasia was observed. GSEA was used to quantify the enrichment of this gene set ($p<0.001$, FDR $Q < 0.001$). Genes were ranked for GSEA using a linear model that takes into

account dysplasia status, pack-years as well as a random variable accounting for batch effects.

Fig. 5A-5C show that Myo-inositol inhibits the PI3K pathway *in vitro*. Insulin was used to activate the PI3K pathway in three different cell lines: (Fig. 5A) BEAS-2B (bronchial airway cell line), (Fig. 5B) BT549 (breast cancer cell line), and (Fig. 5C) HEK293 (human embryonic kidney cell line). The cell lines were then treated with varying doses of myo-inositol and LY-294002. PIP3 levels were measured to quantify the activation levels of the PI3K pathway (y-axis). In each cell line tested, there was a drop in PIP3 levels following treatment with either myo-inositol or LY-294002 (a known PI3K inhibitor), suggesting that myo-inositol inhibits the PI3K pathway *in vitro*. Replication of these experiments produced similar results.

DETAILED DESCRIPTION OF THE INVENTION

Based on the concept that genomic changes in the epithelial cells that line the entire respiratory tract reflect host response to and damage from cigarette smoke, a better understanding of the early events leading to tumorigenesis may be gained by identifying which pathways are deregulated in the airway of smokers with or at risk for having lung cancer. One approach to assess pathway activity uses gene expression data to link *in vitro* activation of an isolated signaling pathway to predict status of that pathway in patient samples. This approach has been successful at predicting pathway status in cell lines as well as tumors where the initiation event is known [13]. A strength of *in vitro* defined pathway signatures is that they are capable of identifying pathway activity at the gene expression level, allowing the measurement of multiple pathways using a single microarray experiment. Further, gene expression based

predictions of pathway activity have been found to correlate significantly to drugs that target the specific pathway [13-20]. Numerous studies have also found correlation of predicted pathway status and therapeutic responsiveness in clinical trials with targeted therapies [22-25].

Work described herein utilized expression signatures developed through *in vitro* perturbation. Metagene models were trained to compare oncogenic pathway activity in the cytologically normal airway epithelium of smokers with and without lung cancer. As described herein, using the gene-expression based pathway approach described above, the data show that the PI3K pathway has an increased level of activity in cytologically normal bronchial airway cells of smokers with lung cancer, as well as higher levels in the lung tumor tissue itself. Biochemical assays measuring *in vivo* PI3K activity in a prospectively collected cohort of airway samples from patients with and without lung cancer validated the computational predictions. An exploration of the expression profiles from the cytologically normal airway of high-risk smokers with dysplastic lesions in their airway again revealed an increased activity of PI3K. As dysplasia is considered a pre-neoplastic event, this is suggestive that PI3K levels increased before the development of lung cancer. Providing possible therapeutic relevance to this result, high-risk subjects responsive to the chemoprophylactic agent myo-inositol show a significant reduction in PI3K activity and regression of dysplastic lesions. The relationship between myo-inositol and PI3K was further elucidated by showing that myo-inositol inhibits PI3K *in vitro*.

Together, these results demonstrate that the PI3K pathway is activated in the cytologically normal airway epithelium prior to the development of lung cancer, and the

levels of this pathway associate with response to chemoprophylaxis with myo-inositol. More broadly, these findings suggest that airway gene-expression reflects perturbation of specific oncogenic pathways within a smoker, potentially allowing for personalized approaches to chemoprophylaxis and therapy.

PI3K and ΔNp63 pathways have an increased activation in the normal airway of smokers with lung cancer (Figure 1A). This is an intriguing finding, because *a priori* cytologically normal cells are not expected to show signs of oncogenic pathway deregulation. Additionally, it is important to note that non-lung cancer controls used as described herein have an extensive range of alternative pathologies that could also impact the PI3K pathway, and are not just healthy volunteers. However, the increased activity is not correlated to smoking status or COPD (Figure 1B, 1C).

Pre-neoplastic increases in the PI3K pathway were also seen in the cytologically normal airway of high-risk smokers with dysplastic airway lesions when compared to healthy smokers. This supports the hypothesis that PI3K activity is induced prior to the development of lung neoplasms. In addition, there are higher levels of PI3K activity in lung tumors as compared to adjacent normal tissue (Figure 2), suggesting a further increase in PI3K activity as cells transform.

Previous studies using mouse models of lung adenocarcinoma have shown that PI3K is required for malignant progression in lung cancer, and that inhibition of this pathway blocks tumorigenesis [40]. Increased activity of the PI3K pathway has also previously been observed in many different cancers, including lung cancer [41]. Some of the common causes of deregulation that confer constitutive

activation in tumors include a mutation in the tyrosine kinase domain of EGFR; a mutation, deletion or suppression of the tumor suppressor PTEN; increased PI3K gene copy number [42] or a mutation in p110α, the catalytic subunit of PI3K [41].

In the studies described herein, PI3K activity in the normal airway epithelium of lung cancer patients is positively correlated with activation of IGF1R (upstream) and PKC (downstream), and is not positively correlated with HER2 (upstream) and AKT (downstream) (Figure 3). These results suggest a specific signaling cascade leading to PI3K activation and subsequent downstream effects in these cells. Increased levels of IGF signaling have been associated with lung cancer in some studies. Further, current inhibitors of the IGF pathway have been found to have significant responses in lung cancer patients. PKC is a kinase downstream of PI3K, and has previously been found to have increased levels in dysplastic lesions and lung cancer [33, 34]. Together, results described herein implicate the IGFR1/PI3K/PKC pathway as central to lung cancer development, even at a pre-malignant state.

Of clinical importance is whether a reduction in PI3K levels prior to the development of lung cancer would offer any therapeutic potential. Current PI3K pathway inhibitors on the market, such as sirolimus, have harmful side effects that would prohibit their use as a long-term prevention option. To help address this crucial question, a study was conducted on a high-risk cohort that had undergone treatment with a lung cancer chemoprophylactic agent called myo-inositol, which has previously been found to reduce dysplastic lesions in the airway following oral treatment for 2-3 months [35]. In contrast to sirolimus, myo-inositol has the potential to be taken orally for long periods of

time because it causes very minor side effects. In patients from this cohort that had responded to myo-inositol as evidenced by a regression of dysplasia in their airway, gene expression patterns were observed that reflect a reduction in PI3K activity. Given the relatively limited samples size for this study (n=10), *in vitro* studies were conducted, and it was found that myo-inositol directly inhibits PI3K activity, suggesting a possible mechanism of action for this compound (Figure 5). If the chemoprophylactic properties of myo-inositol are confirmed in larger clinical trials, use of this compound in high-risk smokers with perturbed PI3K activity in the airway could decrease lung cancer occurrence. Airway gene expression profiling on these subjects post-treatment may also help identify a subset of patients that would benefit from long-term therapy. More broadly, these results suggest that a smoker's pattern of airway gene-expression reflects perturbation of specific oncogenic pathways, potentially allowing for personalized chemoprophylaxis and therapy.

In principal, there are multiple hypotheses that could explain the increased activity of oncogenic pathways in cytologically normal airway epithelium. Importantly, these concepts are not mutually exclusive and likely work in a synergistic manner to promote disease. First, deregulation could be caused by a genetic predisposition to lung cancer, such as oncogenic germ-line mutations. Second, following the field of injury hypothesis, cigarette smoke exposure damages the entire respiratory tract, and the damage, such as somatic mutations, could be the source of oncogenic activity in the airway. The susceptibility to damage will partly depend on host response to cigarette smoke, which will be influenced by oncogenic germ-line mutations. Finally, somatic mutations conferring growth advantages could cause

increased oncogenic activity in the airway due to clonal expansion.

Cumulatively, these studies successfully use a computational approach to identify the signaling pathways driving lung cancer oncogenesis, and to identify rational targeted therapeutic approaches which may be preventative of cancer development in high-risk populations. Further, our biochemical measurements correlate with computational analysis in patient samples, and highlight the specificity and sensitivity of this approach. Verification of pathway activation is also seen in *in vitro* and *in vivo* studies linking myo-inositol treatment to inhibition of the PI3K pathway. This suggests that the deregulation of the PI3K pathway is an early, measurable and reversible step in the development of lung cancer and may serve to guide chemopreventative approaches in high-risk smokers.

Accordingly the invention provides a general approach to identifying pathway status (e.g., oncogenic pathway status) in cytologically normal cells of the airway which may be useful as an early predictor of lung disease and/or which may provide targets for therapeutic intervention (e.g., early intervention). According to this approach, oncogenic or other pathways of interest are activated in a cell (e.g., human epithelial cell, human primary epithelial cell culture) *in vitro* to identify gene expression signatures or patterns which are associated with pathway activation. For example, cells can be perturbed using an adenovirus expressing an activating or necessary component of the pathway of interest (e.g., an adenovirus expressing p110 or other suitable agent). Differentially classified samples (e.g., a sample from a lung cancer patient v. sample from an individual without lung cancer, a sample from a treatment responsive patient v. a sample from a treatment

non-responsive patient, etc.) can then be assessed to identify class associations with a gene expression profile indicative of pathway activation. That is, pathway status (e.g., activation) is correlated with phenotype (e.g., disease state, treatment response, etc.). Thereafter histologically normal airway cells can be tested to identify a gene expression pattern associated with activation of a particular pathway, and based on the correlation between pathway status and phenotype, the phenotype (e.g., disease state such as cancerous, non-cancerous) of the individual from whom the cell sample is obtained can be predicted. In this manner associations between disease state and pathway status (as indicated by gene expression) can be identified, and these associations can be leveraged in therapeutic and prognostic applications. Similarly, the impact of candidate agents and treatment regimens on activation of one or more pathways can be assessed by monitoring gene expression associated with said pathway(s) to identify agents and/or regimens having a desired effect.

The invention also relates to a method of identifying an individual at increased risk of lung disease, comprising determining the activation status of an oncogenic pathway, e.g., the Np63 and/or PI3K pathway, in a cytologically normal airway epithelial cell from said individual. Activation of, e.g., the Np63 and/or PI3K pathway is indicative that said individual is at increased risk of lung disease as compared with an individual in whom the Np63 and/or PI3K pathway is not activated. In particular embodiments the individual is a smoker or a non-smoker. In other embodiments the lung disease is lung cancer. I some embodiments the activation status of multiple pathways (e.g., oncogenic pathways) is assessed simultaneously.

In one embodiment the activation status of the PI3K pathway is determined using gene expression data for one or more (i.e., 1, 2, 3, 4, 5 or more than 5) biomarkers of the PI3K pathway. For example, in some embodiments at least one of said one or more biomarkers is a gene which is increased upon PI3K activation, and in other embodiments at least one of said one or more biomarkers is a gene which is decreased upon PI3K activation. Combinations of biomarkers which are increased and decreased upon PI3K activation may also be used. In particular embodiments at least one of said one or more biomarkers is a gene which is upstream of PI3K activation, while in other embodiments at least one of said one or more biomarkers is a gene which is downstream of PI3K activation.

In one embodiment of the present invention, the isolated nucleic acid is obtained from a cytologically normal airway epithelial cell and used to evaluate expression of a gene or multiple genes using any method known in the art for measuring gene expression, including analysis of mRNA transcripts as well as analysis of DNA methylation.

Methods for assessing mRNA levels are well known to those skilled in the art. In one preferred embodiment, gene expression can be determined by detection of RNA transcripts, for example by Northern blotting, for example, wherein a preparation of RNA is run on a denaturing agarose gel, and transferred to a suitable support, such as activated cellulose, nitrocellulose or glass or nylon membranes. Labeled (e.g. radiolabeled) cDNA or RNA is then hybridized to the preparation, washed and analyzed using methods well known in the art, such as autoradiography.

Detection of RNA transcripts can further be accomplished using known amplification methods. For example, it is within the scope of the present invention to reverse transcribe mRNA into cDNA followed by polymerase chain reaction (RT-PCR); or, to use a single enzyme for both steps as described in U.S. Pat. No. 5,322,770, or reverse transcribe mRNA into cDNA followed by symmetric gap ligase chain reaction (RT-AGLCR) as described by R. L. Marshall, et al., PCR Methods and Applications 4: 80-84 (1994).

Other known amplification methods which can be utilized herein include but are not limited to the so-called "NASBA" or "3SR" technique described in PNAS USA 87: 1874-1878 (1990) and also described in Nature 350 (No. 6313): 91-92 (1991); Q-beta amplification as described in published European Patent Application (EPA) No. 4544610; strand displacement amplification (as described in G. T. Walker et al., Clin. Chem. 42: 9-13 (1996) and European Patent Application No. 684315; and target mediated amplification, as described by PCT Publication WO 9322461.

In situ hybridization visualization may also be employed, wherein a radioactively labeled antisense RNA probe is hybridized with a thin section of a biopsy sample, washed, cleaved with RNase and exposed to a sensitive emulsion for autoradiography. The samples may be stained with haematoxylin to demonstrate the histological composition of the sample, and dark field imaging with a suitable light filter shows the developed emulsion. Non-radioactive labels such as digoxigenin may also be used.

Alternatively, RNA expression, including MRNA expression, can be detected on a DNA array, chip or a microarray. Oligonucleotides corresponding to a gene(s) of interest are immobilized on a chip which is then hybridized with labeled nucleic acids of a test sample obtained from a

patient. Positive hybridization signal is obtained with the sample containing transcripts of the gene of interest. Methods of preparing DNA arrays and their use are well known in the art. (See, for example U.S. Pat. Nos: 6,618,6796; 6,379,897; 6,664,377; 6,451,536; 548,257; U.S. 20030157485 and Schena et al. 1995 Science 20:467-470; Gerhold et al. 1999 Trends in Biochem. Sci. 24, 168-173; and Lennon et al. 2000 Drug discovery Today 5: 59-65, which are herein incorporated by reference in their entirety). Serial Analysis of Gene Expression (SAGE) can also be performed (See for example U.S. Patent Application 20030215858).

The methods of the present invention can employ solid substrates, including arrays in some preferred embodiments. Methods and techniques applicable to polymer array synthesis have been described in U.S. Ser. No. 09/536,841, WO 00/58516, U.S. Pat. Nos. 5,143,854, 5,242,974, 5,252,743, 5,324,633, 5,384,261, 5,405,783, 5,424,186, 5,451,683, 5,482,867, 5,491,074, 5,527,681, 5,550,215, 5,571,639, 5,578,832, 5,593,839, 5,599,695, 5,624,711, 5,631,734, 5,795,716, 5,831,070, 5,837,832, 5,856,101, 5,858,659, 5,936,324, 5,968,740, 5,974,164, 5,981,185, 5,981,956, 6,025,601, 6,033,860, 6,040,193, 6,090,555, 6,136,269, 6,269,846 and 6,428,752, in PCT Applications Nos. PCT/US99/00730 (International Publication Number WO 99/36760) and PCT/US01/04285, which are all incorporated herein by reference in their entirety for all purposes.

Patents that describe synthesis techniques in specific embodiments include U.S. Pat. Nos. 5,412,087, 6,147,205, 6,262,216, 6,310,189, 5,889,165, and 5,959,098.

Nucleic acid arrays that are useful in the present invention include, but are not limited to those that are commercially available from Affymetrix (Santa Clara, Calif.)

under the brand name GeneChip7. Example arrays are shown on the website at affymetrix.com.

The present invention also contemplates many uses for polymers attached to solid substrates. These uses include gene expression monitoring, profiling, library screening, genotyping and diagnostics. Examples of gene expression monitoring, and profiling methods are shown in U.S. Pat. Nos. 5,800,992, 6,013,449, 6,020,135, 6,033,860, 6,040,138, 6,177,248 and 6,309,822. Examples of genotyping and uses therefore are shown in U.S. Ser. No. 60/319,253, 10/013,598, and U.S. Pat. Nos. 5,856,092, 6,300,063, 5,858,659, 6,284,460, 6,361,947, 6,368,799 and 6,333,179. Other examples of uses are embodied in U.S. Pat. Nos. 5,871,928, 5,902,723, 6,045,996, 5,541,061, and 6,197,506.

To monitor mRNA levels, for example, mRNA is extracted from the biological sample to be tested, reverse transcribed, and fluorescent-labeled cDNA probes are generated. The microarrays capable of hybridizing to the gene of interest are then probed with the labeled cDNA probes, the slides scanned and fluorescence intensity measured. This intensity correlates with the hybridization intensity and expression levels.

In one preferred embodiment, gene expression is measured using quantitative real time PCR. Quantitative real-time PCR refers to a polymerase chain reaction which is monitored, usually by fluorescence, over time during the amplification process, to measure a parameter related to the extent of amplification of a particular sequence. The amount of fluorescence released during the amplification cycle is proportional to the amount of product amplified in each PCR cycle.

The present invention also contemplates sample preparation methods in certain preferred embodiments. Prior

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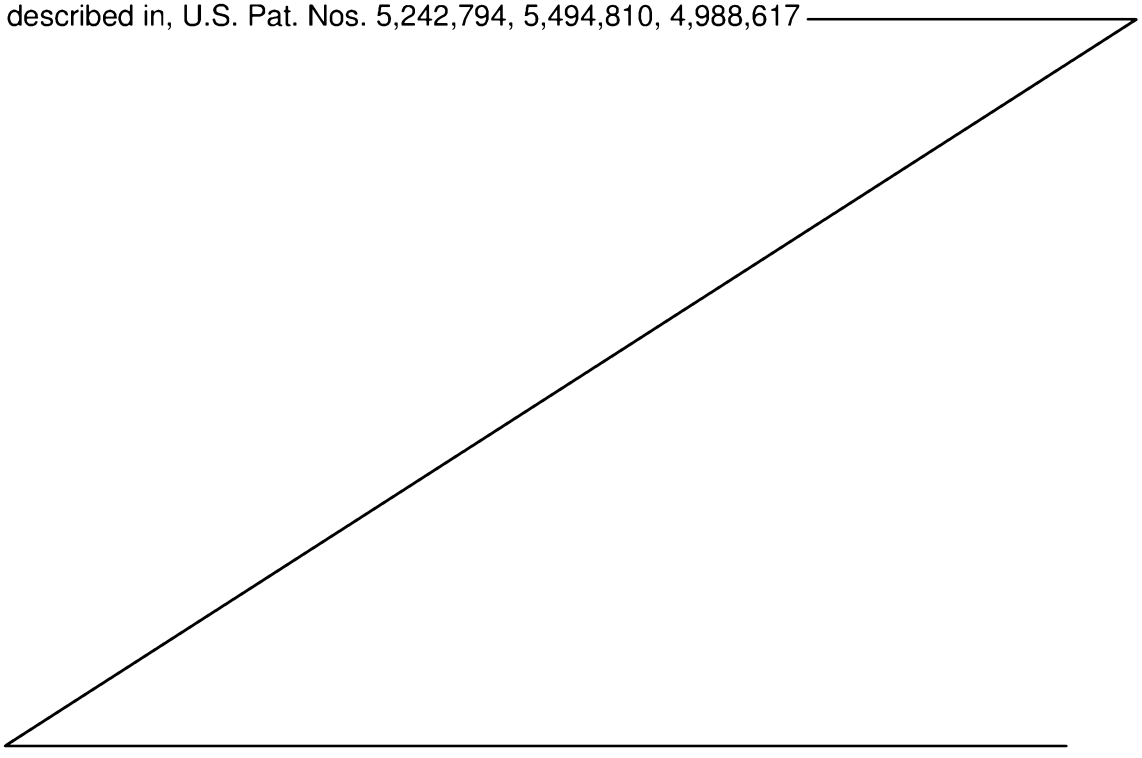
to or concurrent with expression analysis, the nucleic acid sample may be amplified by a variety of mechanisms, some of which may employ PCR. See, e.g., PCR Technology: Principles and Applications for DNA Amplification (Ed. H. A. Erlich, Freeman Press, NY, N.Y., 1992); PCR Protocols: A Guide to Methods and Applications (Eds. Innis, et al., Academic Press, San Diego, Calif., 1990); Mattila et al., Nucleic Acids Res. 19, 4967 (1991); Eckert et al., PCR Methods and Applications 1, 17 (1991); PCR (Eds. McPherson et al., IRL Press, Oxford); and U.S. Pat. Nos. 4,683,202, 4,683,195, 4,800,159 4,965,188, and 5,333,675, and each of which is incorporated herein by reference in their entireties for all purposes. The sample may be amplified on the array. See, for example, U.S. Pat. No 6,300,070, which ise incorporated herein by reference.

Other suitable amplification methods include the ligase chain reaction (LCR) (e.g., Wu and Wallace, Genomics 4, 560 (1989), Landegren et al., Science 241, 1077 (1988) and Barringer et al. Gene 89:117 (1990)), transcription amplification (Kwoh et al., Proc. Natl. Acad. Sci. USA 86, 1173 (1989) and WO88/10315), self-sustained sequence replication (Guatelli et al., Proc. Nat. Acad. Sci. USA, 87, 1874 (1990) and WO90/06995), selective amplification of target polyhucleotide sequences (U.S. Pat. No. 6,410,276), consensus sequence primed polymerase chain reaction (CP-PCR) (U.S. Pat. No. 4,437,975), arbitrarily primed polymerase chain reaction (AP-PCR) (U.S. Pat. Nos. 5,413,909, 5,861,245) and nucleic acid based sequence amplification (NABSA). (See, U.S. Pat. Nos. 5,409,818, 5,554,517, and 6,063,603, each of which is incorporated herein by reference). Other amplification methods that may be used are described in, U.S. Pat. Nos. 5,242,794, 5,494,810, 4,988,617



and in U.S. Ser. No. 09/854,317, each of which is incorporated herein by reference.

Additional methods of sample preparation and techniques for reducing the complexity of a nucleic sample are described, for example, in Dong et al., Genome Research 11, 1418 (2001), in U.S. Pat. Nos. 6,361,947, 6,391,592 and U.S. Patent application Ser. Nos. 09/916,135, 09/920,491, 09/910,292, and 10/013,598.

Methods for conducting polynucleotide hybridization assays have been well developed in the art. Hybridization assay procedures and conditions will vary depending on the application and are selected in accordance with the general binding methods known including those referred to in: Maniatis et al. Molecular Cloning: A Laboratory Manual (2.sup.nd Ed. Cold Spring Harbor, N.Y., 1989); Berger and Kimmel Methods in Enzymology, Vol. 152, Guide to Molecular Cloning Techniques (Academic Press, Inc., San Diego, Calif., 1987); Young and Davism, P.N.A.S, 80: 1194 (1983). Methods and apparatus for carrying out repeated and controlled hybridization reactions have been described, for example, in U.S. Pat. Nos. 5,871,928, 5,874,219, 6,045,996 and 6,386,749, 6,391,623 each of which are incorporated herein by reference.

The present invention also contemplates signal detection of hybridization between ligands in certain preferred embodiments. See, for example, U.S. Pat. Nos. 5,143,854, 5,578,832; 5,631,734; 5,834,758; 5,936,324; 5,981,956; 6,025,601; 6,141,096; 6,185,030; 6,201,639; 6,218,803; and 6,225,625, in provisional U.S. Patent application 60/364,731 and in PCT Application PCT/US99/06097 published as WO99/47964), each of which also is hereby incorporated by reference in its entirety for all purposes.

Examples of methods and apparatus for signal detection and processing of intensity data are disclosed in, for example, U.S. Pat. Nos. 5,143,854, 5,547,839, 5,578,832, 5,631,734, 5,800,992, 5,834,758; 5,856,092, 5,902,723, 5,936,324, 5,981,956, 6,025,601, 6,090,555, 6,141,096, 6,185,030, 6,201,639; 6,218,803; and 6,225,625, in U.S. Patent application 60/364,731 and in PCT Application PCT/US99/06097 (published as WO99/47964), each of which also is hereby incorporated by reference in its entirety for all purposes.

The practice of the present invention may also employ conventional biology methods, software and systems. Computer software products of the invention typically include computer readable medium having computer-executable instructions for performing the logic steps of the method of the invention. Suitable computer readable medium include floppy disk, CD-ROM/DVD/DVD-ROM, hard-disk drive, flash memory, ROM/RAM, magnetic tapes and etc. The computer executable instructions may be written in a suitable computer language or combination of several languages. Basic computational biology methods are described in, e.g. Setubal and Meidanis et al., Introduction to Computational Biology Methods (PWS Publishing Company, Boston, 1997); Salzberg, Searles, Kasif, (Ed.), Computational Methods in Molecular Biology, (Elsevier, Amsterdam, 1998); Rashidi and Buehler, Bioinformatics Basics: Application in Biological Science and Medicine (CRC Press, London, 2000) and Ouelette and Bzevansi Bioinformatics: A Practical Guide for Analysis of Gene and Proteins (Wiley & Sons, Inc., 2.sup.nd ed., 2001).

The present invention also makes use of various computer program products and software for a variety of purposes, such as probe design, management of data, analysis, and instrument operation. See, for example, U.S.

Pat. Nos. 5,593,839, 5,795,716, 5,733,729, 5,974,164, 6,066,454, 6,090,555, 6,185,561, 6,188,783, 6,223,127, 6,229,911 and 6,308,170.

Additionally, the present invention may have preferred embodiments that include methods for providing genetic information over networks such as the Internet as shown in, for example, U.S. patent applications Ser. No. 10/063,559, 60/349,546, 60/376,003, 60/394,574, 60/403,381.

Throughout this specification, various aspects of this invention are presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range. In addition, the fractional ranges are also included in the exemplified amounts that are described. Therefore, for example, a range between 1-3 includes fractions such as 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, etc.

In other embodiments the activation status of the PI3K pathway is determined using one or more gene expression products of one or more biomarkers of the PI3K pathway. Said gene expression products may be nucleotide or amino acid products and can be detected using methods known in the art.

In some embodiments of the invention the activation status of the PI3K pathway is determined by assessing the activation of IGF1R, wherein activation of IGF1R is indicative of activation of the PI3K pathway. In other embodiments of the invention the activation status of the PI3K pathway is determined by assessing the activation of PKC, wherein activation of PKC is indicative of activation of the PI3K pathway. In some embodiments of the invention the activation status of the PI3K pathway is determined by assessing the expression of one or more biomarkers for the PI3K pathway disclosed in [29], the teachings of which are incorporated herein by reference.

In one embodiment the activation status of the Np63 pathway is determined using gene expression data for one or more biomarkers of the Np63 pathway. For example, in some embodiments at least one of said one or more biomarkers is a gene which is increased upon Np63 activation, and in other embodiments at least one of said one or more biomarkers is a gene which is decreased upon Np63 activation. Combinations of biomarkers which are increased and decreased upon Np63 activation may also be used. In particular embodiments at least one of said one or more biomarkers is a gene which is upstream of Np63 activation, while in other embodiments at least one of said one or more biomarkers is a gene which is downstream of Np63 activation.

In particular embodiments, expression data for said one or more biomarkers of the Np63 pathway is obtained using an oligonucleotide microarray. In other embodiments the activation status of the Np63 pathway is determined using one or more gene expression products of one or more biomarkers of the Np63 pathway. Said gene expression products may be nucleotide or amino acid products and can be detected using methods known in the art.

The invention also relates to a method of identifying an individual at increased risk of lung disease, comprising determining the activation status of PKC in a cytologically normal airway epithelial cell from said individual, wherein activation of PKC is indicative that said individual is at increased risk of lung disease as compared with an individual in whom PKC is not activated.

The invention further relates to a method of identifying an individual at increased risk of lung disease, comprising determining the activation status of IGF1R in a cytologically normal airway epithelial cell from said individual, wherein activation of IGF1R is indicative that said individual is at increased risk of lung disease as compared with an individual in whom IGF1R is not activated.

In other embodiments the invention provides an oligonucleotide array having immobilized thereon one or more probes for one or more biomarkers of the PI3K pathway, and wherein said array does not have immobilized thereon probes for other biomarkers. In preferred embodiments said one or more biomarkers of the PI3K pathway are selected from the group consisting of IGF1R, PKC, the biomarkers disclosed in [29], and combinations thereof.

The invention also relates to a method of reducing the risk of lung disease in an individual comprising administering to an individual at risk of lung disease one or more agents (e.g., one or more agents, regimens or treatments or combinations thereof) which inhibit the PI3K pathway. In particular embodiments the PI3K pathway is activated in said individual prior to administration of said one or more agents. In one embodiment the lung disease is lung cancer. In another embodiment said one or more agents are administered to said individual prophylactically before the development of lung disease.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, nucleic acid chemistry, and immunology, which are well known to those skilled in the art. Such techniques are explained fully in the literature, such as, *Molecular Cloning: A Laboratory Manual*, second edition (Sambrook et al., 1989) and *Molecular Cloning: A Laboratory Manual*, third edition (Sambrook and Russel, 2001), (jointly referred to herein as "Sambrook"); *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987, including supplements through 2001); *PCR: The Polymerase Chain Reaction*, (Mullis et al., eds., 1994); Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York; Harlow and Lane (1999) *Using Antibodies: A Laboratory Manual* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (jointly referred to herein as "Harlow and Lane"), and Beaucage et al. eds., *Current Protocols in Nucleic Acid Chemistry* John Wiley & Sons, Inc., New York, 2000).

The teachings of all references and websites cited herein are incorporated herein by reference in their entirety. The invention will be further described by the following non-limiting exemplary embodiment.

Exemplary Embodiment

Methods

Patient Population

Airway epithelial brushings were collected from current and former smokers under suspicion of lung cancer who were undergoing diagnostic flexible bronchoscopy from four institutions- Boston University Medical Center, Boston Veterans Administration, Lahey Clinic and St. James's

Hospital (previously described in [7], see demographics in Table 1 for samples used in this study). Additional brushes were collected from volunteer healthy current, former and never smokers, as well as smokers with COPD, who were undergoing bronchoscopy (some previously published in [31], demographics in Table 1 and 2). Brushings from the cytologically normal bronchial airway of current and former smokers with airway dysplasia was collected at the University of British Columbia from volunteers who were between 40-74, had ≥30 pack-years of cumulative smoke history and had one or more sites of bronchial dysplasia on autofluorescence bronchoscopy (see demographics in Table 2). A subset of these volunteers were treated with myo-inositol for a period of 2-3 months, and an additional brush was collected from cytologically-normal airway epithelium at the end of the treatment when autofluorescence bronchoscopy and endobronchial biopsy was used to measure changes in dysplasia (n=20 samples, 10 individuals) [35].

Prospective samples used for biochemical validation were collected at both Boston University Medical Center and University of Utah Hospital. Cytologically-normal bronchial airway brushings were collected on subjects undergoing bronchoscopy for clinical suspicion of lung cancer (see Table 3). Subjects were followed post-bronchoscopy until a final diagnosis or lung cancer or an alternative lung pathology was made.

The study was approved by the Institutional Review Board of all participating institutions and all subjects provided written informed consent.

Sample Collection and Processing

Cytologically normal airway epithelial samples from smokers with and without cancer (n=129, GSE4115), as well as

current, former and never smokers (n=104, GSE7895) and smokers with (n=127) and without (n=20) COPD (subset of GSE4115, GSE7895 and GSEYYYY) were collected and hybridized onto Affymetrix HG-U133A microarrays as previously described [7, 31]. For lung tumor and adjacent normal studies, GSE10072 was used [32].

Cytologically normal airway epithelial samples from patients with dysplastic airway lesions at the University of British Columbia were collected before and after 2-3 months of treatment with myo-inositol (n=20, 10 patients with two samples each), as well as six additional samples collected pre-treatment with myo-inositol as part of a dose response study. In that study, bronchial brushing was performed in three separate 6-8th generation bronchial airways using a 1.7 mm diameter bronchial cytology brush (Hobbs Medical, Stafford Springs, CT). The brush was retrieved and immediately immersed in RNALater and kept frozen at -80°C until assayed. Epithelial cell content of representative bronchial brushing samples has been quantitated by cytocentrifugation (ThermoShandon Cytospin, Pittsburgh, PA) of the cell pellet and staining with a cytokeratin antibody (Signet, Dedham MA). The cells in the bronchial brush contained >90% bronchial epithelial cells. At least 1 µg of each sample were later hybridized to Affymetrix Human Exon ST microarrays according to the manufacturer's protocol. Data from exon arrays were normalized using RMA-sketch in the Affymetrix Expression Console software.

Airway samples collected prospectively for biochemical validation were snap frozen in liquid nitrogen. For a subset of patients, additional brushes were collected and hybridized to Affymetrix HG U133A 2.0 chips. Microarrays were MAS5.0 normalized in Affymetrix Expression Console.

All new expression data for this study is available for download at GEO under the accession GSEYYYY.

Oncogenic Pathway Activation Probability Calculation

Oncogenic pathways from a prior study were utilized, and calculated as detailed previously [13]. Briefly, primary mammary epithelial cells were cultured and allowed to grow to quiescence. Cell cultures were then infected with adenovirus constructs for key members of a specific pathway (e.g., p110, the catalytic subunit of PI3K) in order to activate the pathway of interest. Samples from the perturbed and normal cell culture were processed and hybridized onto Affymetrix microarrays in replicate (approximately 10 samples for each pathway) 18 hours after infection. Before statistical analysis, probesets were filtered based on low expression or low variance (lowest 25% of each was removed). A gene signature for each pathway was defined by selecting 200 probesets based on correlation with the class variable (e.g., perturbed vs. GFP control). Training of the metagene model was accomplished using the perturbed and GFP control samples, by first summarizing the pathway signature in the training data using the most dominant component from singular value decomposition (SVD), and then using Bayesian fitting of a probit regression model. This was done for each of the pathways, and each model was applied to samples of interest. Resulting pathway probabilities were scaled between zero and one. To determine whether an oncogenic pathway was differentially activated, first a rank-sum test was performed, and for p-values less than 0.05, a random permutation analysis was performed. During random permutation, gene identifiers in the dataset of interest (e.g., bronchial airway) were randomized, and a p-value from a Wilcoxon rank sum test was calculated to measure

differential activation between class variables (e.g., lung cancer vs. no lung cancer). This was repeated 1,000 times.

Microarray data was initially preprocessed by RMA normalizing, and then corrected for batch effects using DWD [43]. Specifically, to standardize expression data in the development of metagene models, DWD was applied to correct batch effects between the oncogenic pathway signature microarray samples, and bronchial airway microarray samples.

Samples in the prospective series that were run on microarrays in order to compare predicted PI3K activity to biochemical measurements were normalized by mas5 due to the small samples size (n=4). Affymetrix U133A 2.0 chips were used. Pathway activity was calculated using MAS5.0 normalized oncogenic pathway signatures, and no DWD standardization was utilized.

Differences between the metagene model used in this manuscript over the original framework are as follows. Previously, to standardize the training dataset with the samples of interest, singular value decomposition was analyzed across all samples, and the two most dominant components were used in the model. The first component generally explained the variance caused by batch effects between the signature dataset and the dataset of interest, while the second component explained the pathway of interest. To remove the standardization from the model, which also removes influence of the test dataset when training the model, batch correction was done in advance (e.g., using DWD), and SVD was applied to the training and test datasets separately (once for the training set to train the model, and a second time for the dataset of interest to apply the model).

GSEA

Gene Set Enrichment Analysis [30] was calculated using GSEA v2. The genes making up the oncogenic pathway signature for PI3K were used to define a PI3K gene set. Importantly, two gene sets were created, one with genes that are increased upon PI3K activation, and one with genes that are decreased upon PI3K activation. Three different analyses were conducted with GSEA, two of which used microarray data from Affymetrix exon arrays. First, healthy current smokers (n=11) were compared to smokers with dysplasia (n=14). Genes were ranked using the following linear model (as calculated in R):

$$Y = \beta_0 + \beta_1 + \beta_2 + b + \varepsilon$$

Where Y is the expression of a gene, β_0 is the intercept, β_1 measures lung cancer risk (whether the sample has dysplasia or not), β_2 is the cumulative cigarette smoke exposure for each person (pack-years), b is a random effect correcting for batch differences, and ε is the error term. The coefficient of β_1 was used to rank the genes for GSEA. Second, to compare pre- and post-treatment with myo-inositol, samples were ranked using a paired Wilcoxon rank sum test. The GSEA analysis done in the U133A airway dataset used the default signal to noise ranking. 100 gene set permutations were used to calculate FDR.

Kinase Assay

80% confluent BEAS- 2B, BT549, or HEK293 cells were starved in BEBM, RPMI, or DMEM medium, respectively (Clonetics, GibcoBRL). This media contained either 0.1% added supplements for the BEAS-2B cells or 0.1% fetal bovine serum for BT549 and HEK293 cells for 24 hrs. Cells were then pretreated with increased concentrations of myo-inositol (Sigma), or LY294002 (Sigma) for 16 hrs. at 37°C. Prior

stimulation, the cells were treated with fresh drugs for another 30 min, then 500 uM of insulin (SIGMA) were added for 15 minutes at 37°C. The cells were lysed in RIPA buffer (20 mM TRIS (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 1 mM EDTA, 0.1% SDS) containing 0.1 mM sodium orthovanadate, 2 mM PMSF, 100uM protease inhibitors (Sigma). Lysates were centrifuged at 14000 rpm for 20 minutes at 4°C and incubated with monoclonal anti-p85 PI3K (Santa Cruz) antibody for 1 hr at 4°C. The bounded proteins were precipitated with 50 ul of 50 % slurry protein G Sepharose (Sigma) and washed three times with lysis buffer, three times with buffer containing 0.1 mM Tris (pH 7.4), 5 mM LiCl, 0.1 mM sodium orthovanadate, and two times with buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.1 mM sodium orthovanadate. The beads were washed in kinase buffer (50 mM Tris (pH 7.4), 10 mM $MgCl_2$) containing 20 uM cold ATP (Sigma), and resuspended in 45 ul of kinase buffer containing 5 ul of L-a- phosphatidylinositol-4,5-bisphosphate (Avanti Polar Lipids) (1 mg/ml), and 20 uCi ATP (32-P) for 20 minutes at RT. The reactions were stopped by addition of 100 ul 1N HCl, and the lipids were extracted with 160 ul of CHCl3/MeOH (1:1). The phosphorylated products were separated by TLC on Silica 60 plates pretreated with potassium oxalate in a CHCl3/MeOH/NH4 solution (45:35:1.5). The production of PIP3 was evaluated by autoradiography and quantified by densitometry analysis and scintillation analysis. All experiments for each cell line were repeated at least twice with similar results.

Western Blot Analysis

Patient tissue samples were collected by bronchoscopy and snap frozen immediately in liquid nitrogen . Cell extracts from bronchoscopy brushes were prepared by adding

200 ul of RIPA buffer. To facilitate the detachment of the cells from the brushes, the tubes were vortexed three times for 5 seconds. Both cell and bronchoscopy brushing extracts were centrifuged at 14000 rpm for 20 minutes at 4°C and the pellets discarded. The protein yield was quantified by Bradford assay, and equivalent amount of protein was loaded to 7% SDS-PAGE gels. The membrane were blocked for 1 h in blocking buffer (Tris buffer saline containing 0.1% Tween 20 and 2.5% BSA, or Tris buffer saline containing 0.1% of Tween 20 and 5% low fat milk), and placed in primary antibody (Tris buffer saline containing 0.1% Tween 20 and 2.5% BSA, 0.02% sodium azide) overnight at 4°C. The primary antibodies used in this study are the followed: rabbit phospho-PKC (pan)(βII Ser660) ratio 1:100 (Cell Signaling Techn.); rabbit phospho-IGF-I Receptor β (Tyr1131)/Insulin Receptor β(Tyr 1146) ratio 1:100 (Cell Signaling Techn.); rabbit phospho-PLCγ1 (Tyr783), ratio 1:500 (Cell Signaling Techn.); rabbit phospho-AKT (Ser473) ratio 1:100 (Cell Signaling Techn.); goat PI3-Kinase p110α (C17) ratio 1:50 (Santa Cruz), rabbit phospho-ERK ratio 1:100 (Cell Signaling) rabbit GAPDH , ratio 1:1000 (AbCam). Nitrocellulose were washed three times in Tris buffer saline containing 0.1% Tween 20 and/or 0.1% NP-40. Primary antibody was detected using horseradish peroxidase-linked secondary antibody and visualized with the ECL Plus Western Blot Detection system (GE Healthcare).

Results

PI3K pathway activation in cytologically normal bronchial airway epithelial cells of smokers with lung cancer

Cytologically normal bronchial airway epithelial cell brushings were obtained from current and former smokers undergoing flexible bronchoscopy for suspicion of lung

cancer and were hybridized to DNA microarrays as previously described [7] (n=129, see patient demographics in Table 1). To help elucidate oncogenic pathway signaling changes in these cells, we utilized a previously published gene expression dataset and computational approach [13, 26-28]. Oncogenic pathway signatures [13] were experimentally derived by activating a pathway via expression of a specific oncogene in primary human epithelial cells. A gene expression signature was then defined by identifying which genes are altered following pathway activation, and used to predict pathway activity in other in vivo samples. Using this methodology, oncogenic pathway activation probabilities for seven signaling pathways (Ras, Myc, E2F3, Src, β-catenin, ΔNp63 and phosphatidylinositol 3' kinase (PI3K)) were calculated for the bronchial airway epithelial of current and former smokers with suspicion for lung cancer[7]. It is important to note that although approximately half of these patients were ultimately diagnosed with primary lung cancer (the remainder were found to have alternate lung pathologies), the brushings collected from the proximal mainstem bronchus (i.e. not adjacent to the tumor or lung lesion), were cytologically normal and were >90% epithelial. Thus, *a priori* one would not expect differential oncogenic pathway activity in the normal airway of smokers with lung cancer.

Of the seven pathways tested, only two were found to be significantly and differentially activated in the airway of smokers with lung cancer compared to controls with alternate lung pathologies after a random permutation analysis: ΔNp63 and PI3K ($p<0.001$, Figure 1A). Furthermore, the genes that have been found to play roles in the phosphatidylinositol signaling system pathway [29, the teachings of which are incorporated by reference herein] were also found to be

significantly upregulated in lung cancer patients using Gene Set Enrichment Analysis [30] (GSEA, p=0.034, FDR q=0.099). Upregulation of PI3K was not limited to a specific cancer cell type, tumor location or tumor stage (data not shown).

PI3K activation is not significantly correlated to cumulative smoke exposure or COPD

We next sought to determine whether the increased pathway activity of PI3K was due to the presence of cancer in the lung, or caused by other confounding factors such as differences in cumulative smoke exposure (patients with cancer had higher cumulative exposure, see Table 1) or other pulmonary diseases. First, an ANCOVA using cumulative smoke exposure as a covariate was used to test for differential pathway activation between patients with and without lung cancer. PI3K ($p = 2.08x10-8$) remained significantly differentially activated after addressing the possible confounding influence of differences in tobacco exposure. Second, using a whole-genome gene expression dataset of bronchial airway epithelium collected from current (n=52), former (n=31) and never (n=21) healthy smokers [31], we calculated the pathway activation probabilities for all seven pathways using the same methodologies previously described. While none of the seven pathways were differentially activated between healthy current, never and former smokers, the ΔNp63 pathway trended towards being significantly activated when comparing never and current smokers (p=0.09, Figure 1B). Finally, using a bronchial airway gene expression dataset obtained from smokers with (n=17) and without (n=20) chronic obstructive pulmonary disease (COPD), Ras was the only pathway that was differentially activated ($p<0.001$, data not shown) (Figure 1C). This lends evidence that the significant differential

activation of the oncogenic pathway PI3K in the normal bronchial airway is specific to individuals with lung cancer, though the mechanism of differential activation is unknown. Due to the known deregulation of p53 related pathways (such as ΔNp63) in response to environmental stressors, as well as the lack of any known therapeutic modulators of the p53/p63 pathway, we chose to focus on the PI3K pathway for all further studies.

PI3K is activated in lung cancer tissue

The deregulation of PI3K in cytologically normal airway epithelial cells of patients with lung cancer led us to examine PI3K activity in lung cancer tissue. We would expect a significant increase in oncogenic pathways essential for tumor growth and survival during lung cancer development. For this analysis, we used a published dataset comprised of lung adenocarcinoma and matched adjacent non-tumor tissue (n=107) [32]. Pathway status was predicted using the same genomic approach detailed above. As shown in Figure 2, malignant lung tumors had a highly significant ($p<0.001$) increase in PI3K activity as compared to the adjacent non-tumor tissue. ΔNp63 pathway was also increased to a lesser extent in tumor cells ($p=0.002$). This result highlights a central role for the PI3K pathway in the malignant progression of cells, and supports our hypothesis that PI3K activation is important for lung cancer tumorigenesis. A significant increase was also seen for the Myc, E2F3 and Src pathways in lung tumors, confirming their roles in lung tumor growth and development. As lung tumors are comprised of dividing cells and have higher levels of proliferation than normal tissue, we would expect pathway involved in cell growth (Myc, E2F3, Src) to also be increased in this analysis.

Biochemical analysis of PI3K pathway activity in the bronchial airway

To validate our gene expression findings, we measured PI3K enzymatic activity in a prospectively collected cohort of cytologically-normal airway epithelial samples from subjects undergoing bronchoscopy for clinical suspicion of lung cancer. Samples were independently obtained from Boston University Medical Center and University of Utah Hospital between October 2007 and June 2008. Subjects were followed post-bronchoscopy until a final diagnosis of lung cancer or an alternate lung pathology was made. Importantly, subjects without lung cancer had a range of other pathologies, including metastatic cancer of non-lung origin, sarcoidosis, septic emboli, and pneumonia. Following protein extraction from the airway brushings, a PI3K kinase assay was performed. Based on our genomic predictions, we would expect a majority of samples from patients with cancer to have high PI3K activity and only a minority of the samples from patients with alternative pathologies to have high PI3K activity.

As seen in Figure 3A, PI3K showed increased activation in the majority of patients with lung cancer (70% of the lung cancer samples in the genomic analysis were in the top half of PI3K activity), as compared to patients without lung cancer (30%). Specifically, we see high correlation between PI3K activity and lung cancer status for both cohorts (Boston: R = 0.499, Utah: R = 0.389). For a subset of these samples (n=4), we were able to collect additional bronchial epithelial cells to perform microarray analysis and predict PI3K activity using the same approach as was used on the original dataset. Predicted PI3K activity was correlated with the PI3K levels measured biochemically within the same

individual (R=0.48). Together, these results support the conclusion that PI3K activity is increased in the normal airway of patients with lung cancer and validate our computational predictions of PI3K activity calculated from gene expression data. These results are even more striking when considering the variety of pathologies in the control patients that might also affect PI3K activity.

In an attempt to further define the causes and consequences of elevated PI3K activity, we performed Western Blotting analysis of the phosphorylation state of key PI3K pathway components. Phosphorylation of IGF1R (Boston: R= 0.91, Utah: R= 0.34), but not HER2 (Boston R= -0.61, Utah R= -0.41), positively correlated to PI3K kinase activity (Figure 3B), suggestive of this receptor being an upstream effector for PI3K activation in the normal airway epithelial tissue of patients with lung cancer. Correlation between biochemically measured PI3K activity and IGF1R in patients with lung cancer shows a high positive correlation, further supporting a potential relationship between IGF1R activation and PI3K activity (Boston R= 0.98, Utah R= 0.90) (Figure 3B). Next, measured phosphorylation of Akt and PKC, the major downstream effectors of PI3K, was measured. PKC had higher correlation with biochemically measured PI3K activity in patients with lung cancer, while no significant correlation was seen for Akt (Figure 3B), again highlighting activation of specific PI3K pathways in patients with lung cancer. Cumulatively, these results support our genomic findings that PI3K activity is enriched in the normal lung cells of patients with lung cancer compared to patients with other pathologies, and the activation of IGF1R is a potential mechanism for this activity [33, 34].

PI3K pathway activation in high-risk smokers with dysplasia

To evaluate the hypothesis that an increased activation of PI3K is an early event in the development of lung cancer, we compared gene expression in cytologically normal airway epithelia from a group of healthy smokers (n=11) to a group of smokers with moderate-severe airway dysplasia (n=14, from [35]) (See Table 2). As dysplasia is considered to be a pre-neoplastic event, this cohort represents "high-risk" smokers who have an increased likelihood of getting lung cancer [36, 37]. If high-risk smokers with dysplasia have higher levels of PI3K than healthy smokers, it would suggest that the increased activity in the airway epithelium precede the development of lung cancer. The high-risk cohort, as well as a cohort of healthy current smokers, was hybridized to Affymetrix Human Exon arrays. Due to platform differences with the oncogenic pathway signatures, we were unable to use the metagene model to calculate pathway activity. Instead, GSEA was used to compare PI3K activity between the two groups, and the PI3K gene signature defined by the *in vitro* experiments was split into two gene sets defined by the genes that go up or down with PI3K activation (PI3K_Up, PI3K_Down). Given that there were significant differences in cumulative tobacco exposure between the two groups, a linear model incorporating both batch effects and pack-years was used to rank the genes for use in GSEA. A significant increase in PI3K activity in the cytologically normal airway of subjects with dysplasia was found ($p<0.001$, FDR $Q=0.022$ and $p<0.001$, $Q<0.001$ for PI3K_Up and PI3K_Down gene sets respectively) (Figure 4). The increased activity of PI3K in the airway of people with dysplasia indicates that the deregulation of PI3K is an early event in lung cancer tumorigenesis.

Reversibility of PI3K activity in high-risk smokers treated with myo-inositol

Given elevated PI3K activity in both high-risk smokers as well as smokers with lung cancer, we next wanted to determine if a decrease of PI3K activity would correlate with regression of dysplastic lesions. A recent phase 1 clinical study published by Lam et al. [35] studied myo-inositol as a lung cancer chemoprophylactic agent in high-risk smokers. In this study, volunteer smokers with ≥30 pack-years of smoking history were screened for the presence of dysplasia in their airway using autofluorescent bronchoscopy. Ten current and former smokers with moderate-severe dysplasia documented on endobronchial biopsy were then given oral myo-inositol for 2-3 months, and the status of their dysplastic lesions was again measured by repeat endobronchial biopsy of the site. When compared to control patients who were treated with placebo, myo-inositol was found to significantly increase the rate of dysplastic regression. Airway brushings of cytologically normal bronchial epithelium were also collected both before and after treatment, and gene expression profiling was performed (see demographics in Table 2).

GSEA analysis was again used to measure changes in the expression of genes in the PI3K pathway. When comparing before treatment with myo-inositol versus after treatment, subjects who did respond to treatment (n=6, 12 samples) showed increased expression of genes in the PI3K Down gene set (p=0.04, FDR Q=0.177), which is suggestive of a decrease in PI3K levels following treatment with myo-inositol. Those that did not respond to treatment (n=3, 6 samples) had no change in the levels of the PI3K gene sets. The decrease in airway PI3K activity seen in patients who respond to myo-

inositol demonstrates that regression of dysplasia is correlated to the activity level of this pathway.

Myo-inositol as a PI3K inhibitor

A reduction in PI3K activity in patients that responded to myo-inositol with a regression of dysplasia in their airway reinforces the association between elevated PI3K activity levels and the presence of pre-neoplastic airway lesions. However, the mechanism of action of myo-inositol has remained undefined, and the study was limited by a relatively small sample size. To further explore the relationship between myo-inositol and the PI3K activity, we tested the ability of myo-inositol to inhibit PI3K in vitro. Following activation of PI3K by treating cells with insulin, cells were treated with increasing doses of either myo-inositol or LY-294002 (a known PI3K inhibitor). PI3K activity, as measured by PIP3 levels, was then quantified using a standard kinase assay protocol [38, 39]. For this experiment, three different cell lines were analyzed in replicate: BEAS-2B (airway), BT549 (breast cancer) and HEK293 (embryonic kidney). In all three cell lines, myo-inositol inhibited PI3K activity levels in a dose-dependent manner (Figure 5). Thus, myo-inositol is an inhibitor of PI3K, and has chemoprophylactic properties associated with regression of dysplasia in airway epithelial cells.

Tables:

	Primary Dataset		Never/Former/Current Smoker Dataset			COPD Dataset	
	Lung Cancer	*No Lung Cancer*	*Never*	*Former*	*Current*	*No COPD*	*COPD*
Samples	60	69	21	31	52	20	17
Age	64.1 (9)*	49.8 (15.2)*	32.3 (10.7)*	55.9 (14.7)*	48.6 (15.2)*	43.6 (9.3)*	60.4 (12.3)*
Pyers	57.4 (25.6)*	29.4 (27.3)*		34.0 (30.1)	34.5 (34.2)	33.5 (25.9)*	57.4 (27.9)*
Smoking Status	51.7% F, 48.3% C	37.3% F, 62.3% C		100% F	100% C	100% C	100% C
Months Since Quit (Formers)	113 (118)	158 (159)		145.2 (162.8)			

* Feature is significantly different between the classes of that dataset ($p<0.05$)

Table 1. Patient demographics for the Affymetrix U133A platform datasets used in this study. These cohorts were used to explore differences in oncogenic activity in the cytologically normal airway epithelium of smokers with lung cancer. Average number for clinical variables is shown, with the standard deviation in parenthesis. Under "Smoking Status," C = current smoker, and F = Former smoker. COPD = Chronic Obstructive Pulmonary Disease. All datasets consist of samples collected via brushing of cytologically-normal bronchial airway epithelium obtained during bronchoscopy.

	Smokers With Dysplasia (Myo-inositol dataset)		Healthy Smokers
	Matched Samples Before and After Treatment	*Airway with Dysplasia*	*Current*
Samples	20 (10 before, 10 after)	6	11
Age	61.7(8.3)	64.5 (7.1)	36.5 (10.0)
Pack-Years	42.9 (23.7)	59.8 (17.7)	12.4 (9.2)
Smoking Status	70% F, 30% C	100% C	100% C
Months Since Quit (Formers)	145.7 (89.9)		

* Age, Pack-years significantly different between dysplasia and healthy smoker datasets ($p<0.05$)

Table 2. Patient demographics for the Affymetrix exon array platform datasets used in this study. These cohorts were used to explore PI3K activity prior to the development of lung cancer, as well as changes in PI3K activity following treatment with a lung cancer chemoprophylaxis agent (myo-inositol). Average number for clinical variables is shown, with the standard deviation in parenthesis. Under "Smoking Status," C = current smoker, and F = former smoker. All datasets consist of samples collected via brushing of cytologically-normal bronchial airway epithelium obtained during bronchoscopy.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. A method of identifying an individual at increased risk of lung disease, comprising determining the activation status of the PI3K pathway in a cytologically normal airway epithelial cell from said individual, wherein activation of the PI3K pathway is indicative that said individual is at increased risk of lung disease as compared with an individual in whom the PI3K pathway is not activated.

2. A method according to claim 1 wherein the individual is a smoker.

3. A method according to claim 1 wherein the individual is a non-smoker.

4. A method according to any one of claims 1 to 3 wherein the lung disease is lung cancer.

5. A method according to any one of claims 1 to 4 wherein the activation status of the PI3K pathway is determined using gene expression data for one or more biomarkers of the PI3K pathway.

6. A method according to claim 5 wherein at least one of said one or more biomarkers is a gene which is increased upon PI3K activation.

7. A method according to claim 5 wherein at least one of said one or more biomarkers is a gene which is decreased upon PI3K activation.

8. A method according to claim 5 wherein at least one of said one or more biomarkers is a gene which is upstream of PI3K activation.

9. A method according to any one of claims 5 to 8 wherein the gene expression data for said one or more biomarkers of the PI3K pathway is obtained using an oligonucleotide microarray.

10. A method according to any one of claims 1 to 4 wherein the activation status of the PI3K pathway is determined using one or more gene expression products of one or more biomarkers of the PI3K pathway.

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11. A method according to any one of claims 1 to 4 wherein the activation status of the PI3K pathway is determined by assessing the activation of IGF1R, wherein activation of IGF1R is indicative of activation of the PI3K pathway.

12. A method according to any one of claims 1 to 4 wherein the activation status of the PI3K pathway is determined by assessing the activation of PKC, wherein activation of PKC is indicative of activation of the PI3K pathway.

13. A method of identifying an individual at increased risk of lung disease, comprising determining the activation status of PKC in a cytologically normal airway epithelial cell from said individual, wherein activation of PKC is indicative that said individual is at increased risk of lung disease as compared with an individual in whom PKC is not activated.

14. A method of identifying an individual at increased risk of lung disease, comprising determining the activation status of IGF1R in a cytologically normal airway epithelial cell from said individual, wherein activation of IGF1R is indicative that said individual is at increased risk of lung disease as compared with an individual in whom IGF1R is not activated.

15. A method according to claim 9 wherein said oligonucleotide microarray has immobilized thereon one or more probes for one or more biomarkers of the PI3K pathway, and wherein said microarray does not have immobilized thereon probes for other biomarkers.

16. A method according to claim 15 wherein said one or more biomarkers of the PI3K pathway are selected from the group consisting of IGF1R, PKC and combinations thereof.

17. A method according to any one of claims 1, 13 or 14, substantially as herein described with reference to any one or more of the examples but excluding comparative examples.

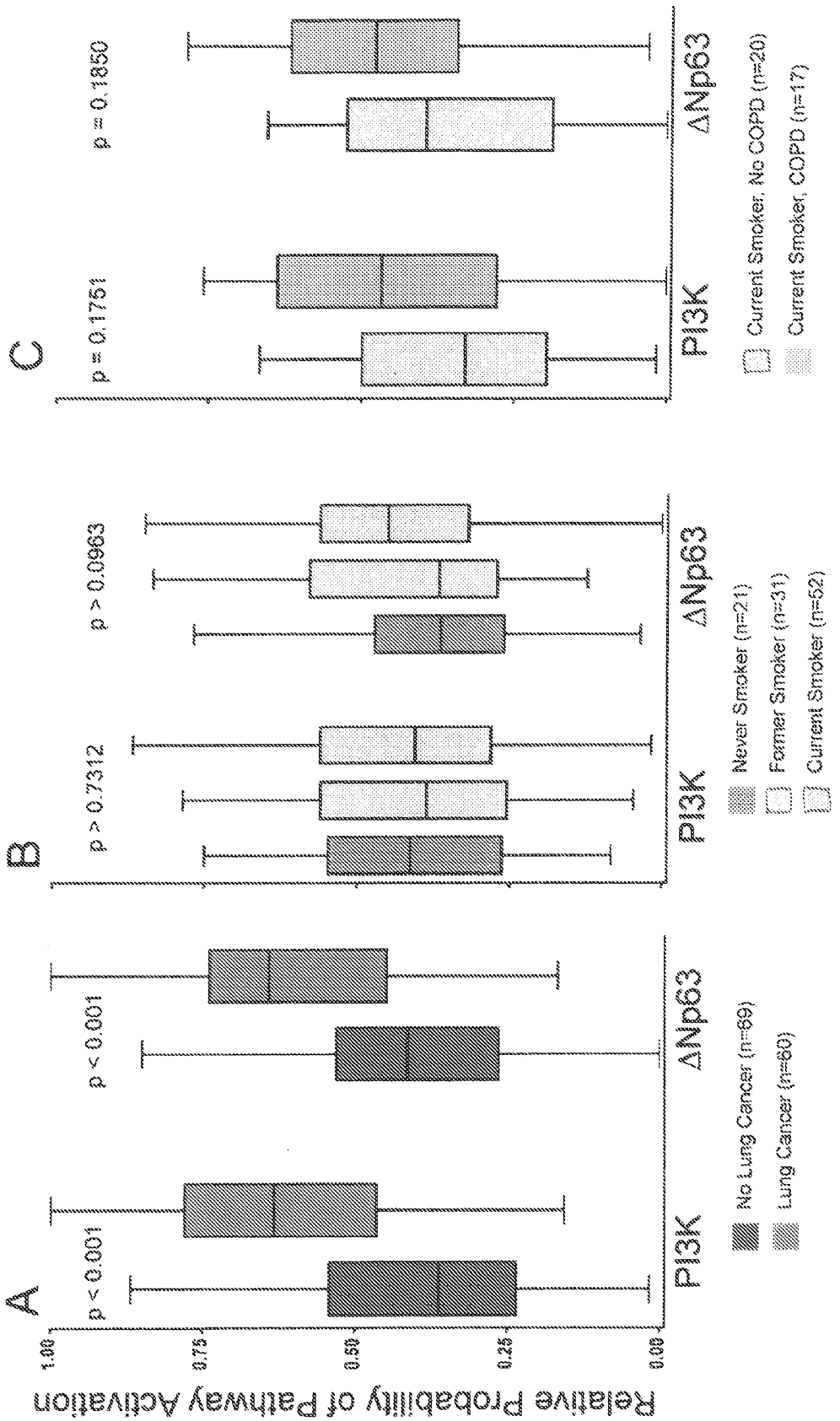
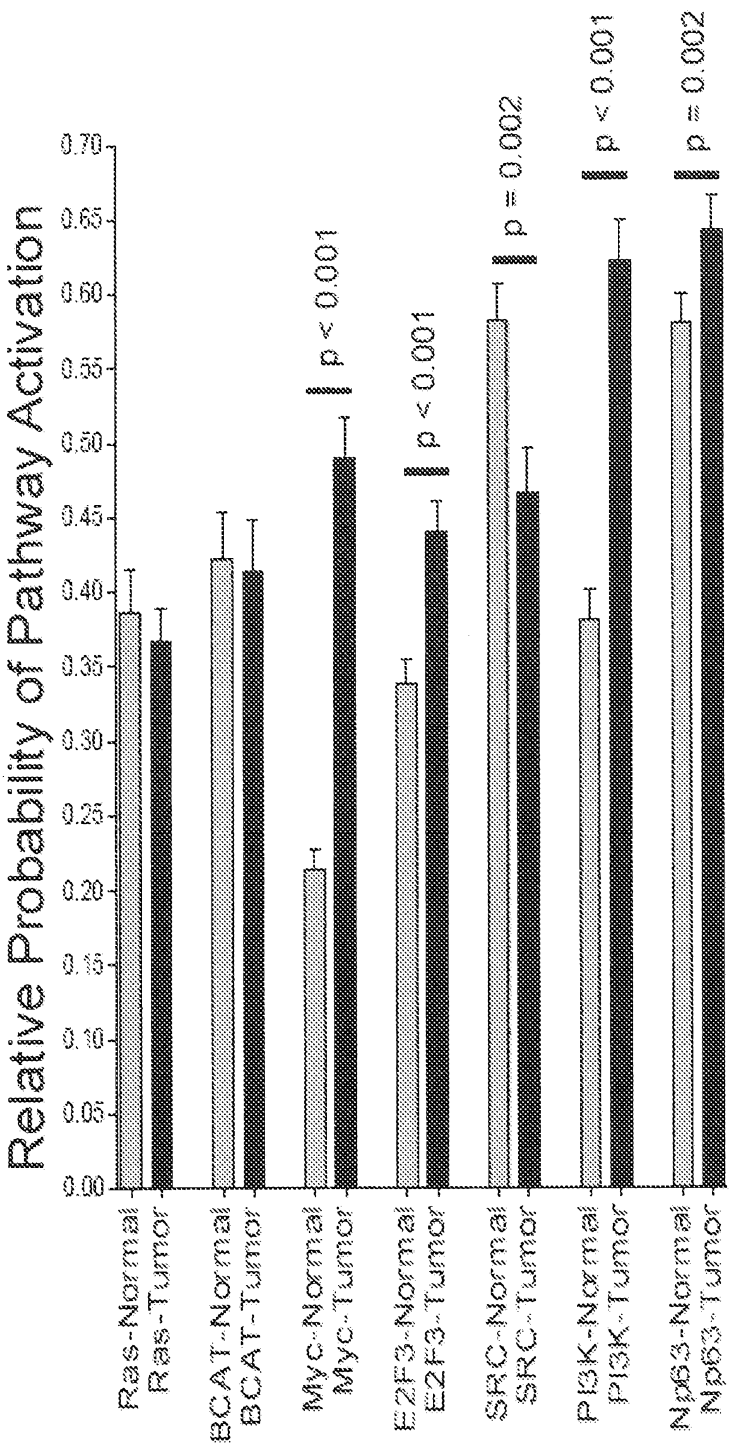

A
B
C
Relative Probability of Pathway Activation
1.00
0.75
0.50
0.25
0.00
p < 0.001
p < 0.001
p > 0.7312
p > 0.0963
p = 0.1751
p = 0.1850
P13K
ΔNp63
No Lung Cancer (n=69)
Lung Cancer (n=80)
Never Smoker (n=21)
Former Smoker (n=31)
Current Smoker (n=52)
Current Smoker, No COPD (n=20)
Current Smoker, COPD (n=17)

Fig. 1A-1C


Relative Probability of Pathway Activation
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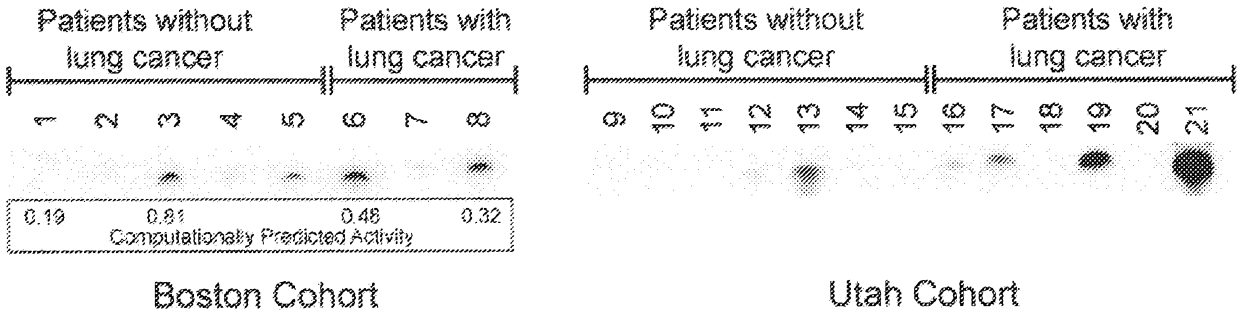
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
p < 0.001
p < 0.001
p = 0.002
p < 0.001
p = 0.002
Ras-Normal
Ras-Tumor
BCAT-Normal
BCAT-Tumor
Myc-Normal
Myc-Tumor
E2F3-Normal
E2F3-Tumor
SRC-Normal
SRC-Tumor
PI3K-Normal
PI3K-Tumor
Np63-Normal
Np63-Tumor

Fig. 2

A. PI3K Kinase Assay



B. Pearson correlation heatmap

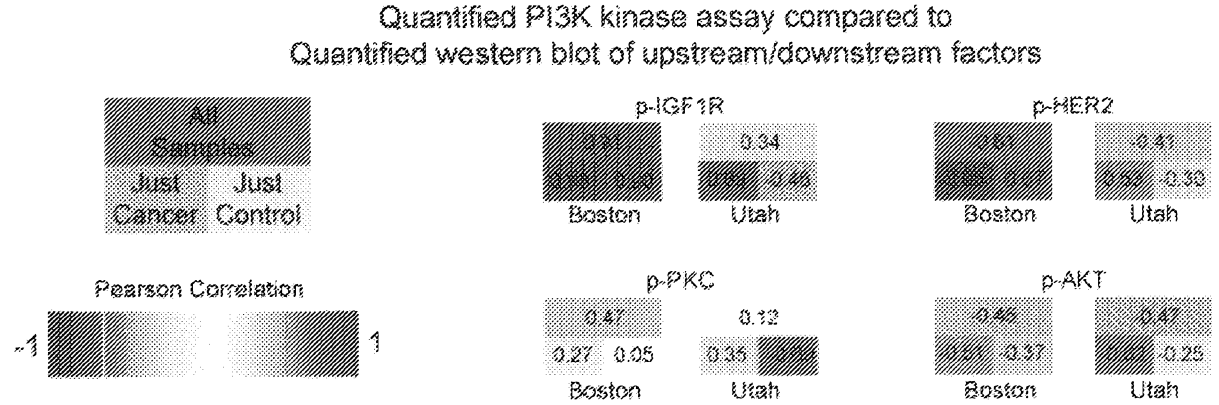


Fig. 3A-3B

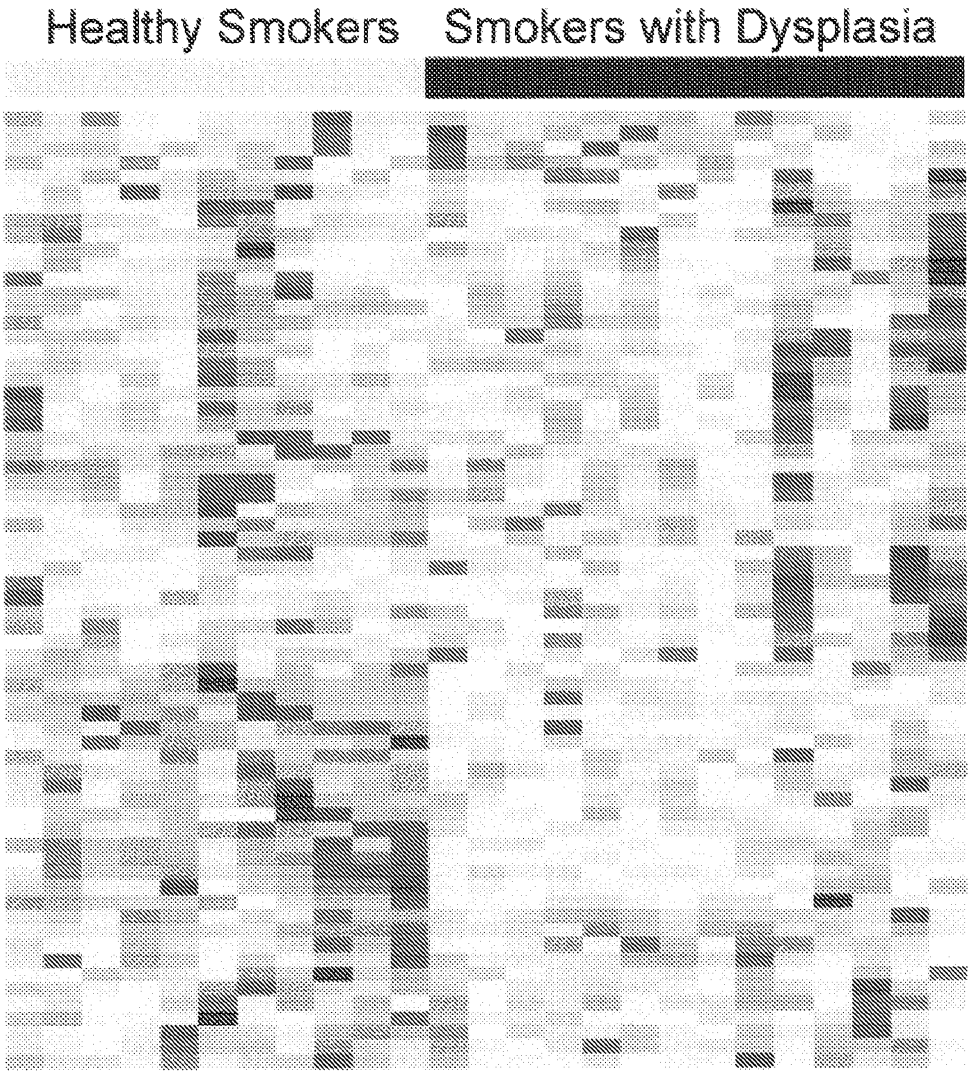
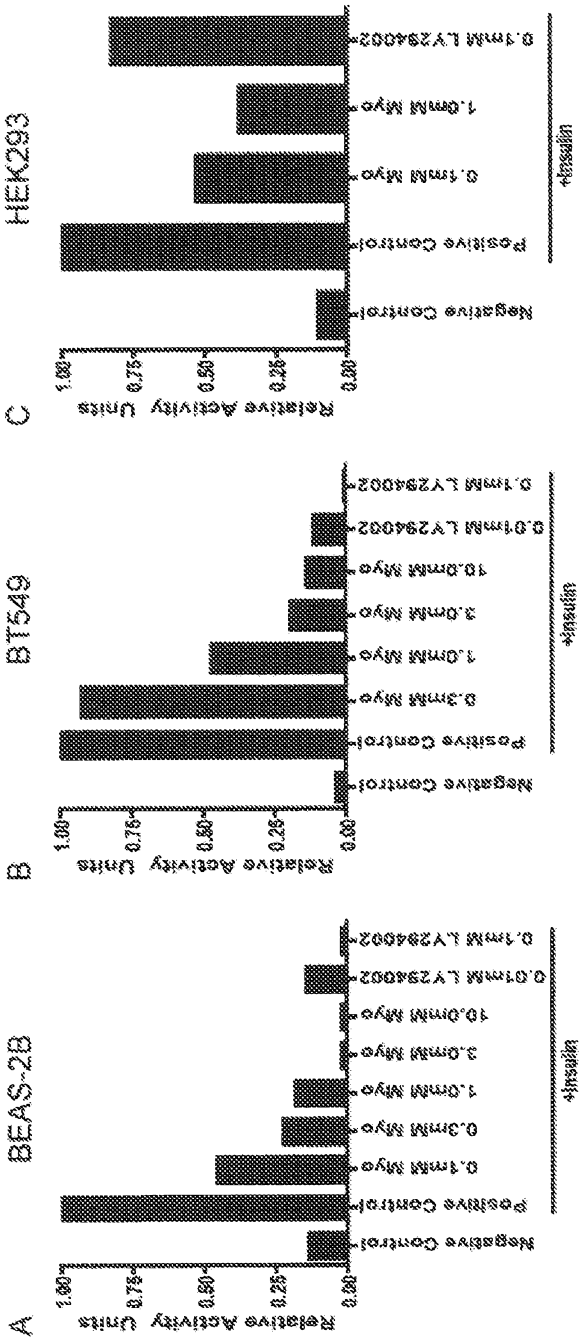

Healthy Smokers
Smokers with Dysplasia
Genes Up-Regulated When PI3K Is Activated
p < 0.01, FDR < 0.01

Fig. 4


A
BEAS-2B
B
BT549
C
HEK293
Relative Activity Units
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0.75
0.50
0.25
0.00
Negative Control
Positive Control
0.1mM Myo
0.3mM Myo
1.0mM Myo
3.0mM Myo
10.0mM Myo
0.01mM LY294002
0.1mM LY294002
+Insulin

Fig. 5A-5C