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Aberrant Signaling Pathways in Squamous Cell Lung Carcinoma

Ivy Shi[1,2], Nooshin Hashemi Sadraei[3], Zhong-Hui Duan[4,5] and Ting Shi[1]

[1]Department of Cancer Biology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH 44195, USA.
[2]Hudson High School, Hudson, OH 44236, [3]Department of Solid Tumor Oncology, Taussig Cancer Institute, Cleveland
Clinic Foundation, Cleveland, OH 44195, USA. [4]Department of Computer Science, University of Akron, Akron,
OH 44236, USA. [5]Program in Integrated Bioscience, University of Akron, Akron, OH 44236, USA.
Corresponding author email: shiti@ccf.org

Abstract: Lung cancer is the second most commonly occurring non-cutaneous cancer in the United States with the highest mortality rate among both men and women. In this study, we utilized three lung cancer microarray datasets generated by previous researchers to identify differentially expressed genes, altered signaling pathways, and assess the involvement of Hedgehog (Hh) pathway. The three datasets contain the expression levels of tens of thousands genes in normal lung tissues and squamous cell lung carcinoma. The datasets were combined and analyzed. The dysregulated genes and altered signaling pathways were identified using statistical methods. We then performed Fisher's exact test on the significance of the association of Hh pathway downstream genes and squamous cell lung carcinoma.

395 genes were found commonly differentially expressed in squamous cell lung carcinoma. The genes encoding fibrous structural protein keratins and cell cycle dependent genes encoding cyclin-dependent kinases were significantly up-regulated while the ones encoding LIM domains were down. Over 100 signaling pathways were implicated in squamous cell lung carcinoma, including cell cycle regulation pathway, p53 tumor-suppressor pathway, IL-8 signaling, Wnt-β-catenin pathway, mTOR signaling and EGF signaling. In addition, 37 out of 223 downstream molecules of Hh pathway were altered. The *P*-value from the Fisher's exact test indicates that Hh signaling is implicated in squamous cell lung carcinoma.

Numerous genes were altered and multiple pathways were dysfunctional in squamous cell lung carcinoma. Many of the altered genes have been implicated in different types of carcinoma while some are organ-specific. Hh signaling is implicated in squamous cell lung cancer, opening the door for exploring new cancer therapeutic treatment using GLI antagonist GANT 61.

Keywords: biomarkers, drug targets, signaling pathways, microarray technology, non-small cell lung cancer, cancer treatment

Introduction

Cancer is the third leading cause of death worldwide, and lung cancer is the second most commonly occurring non-cutaneous cancer with the highest mortality rate among all cancers. There are 1.38 million new cases of lung cancer every year, along with 1.23 million deaths due to lung cancer.[1] Based on the histological types of the cells, lung cancer is categorized into two major types, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC occurs almost exclusively in heavy smokers, and makes up about 14% of all lung cancers. NSCLC is a general term for several different types of lung cancer, including squamous cell carcinoma, in which the malignant cells appear larger in size in comparison to SCLC. Collectively, NSCLC subtypes contribute to about 86% of all lung cancers.[2] The average survival time for patients with SCLC is about 10 months. The survival time for patients with NSCLC varies depending on the stage of the disease. Patients with an advanced stage of NSCLC also have an average survival time of 10 months.[3] The 5-year survival rate for SCLC is only 6%, and for NSCLC, the rate is merely 16%, making the average rate 14%, one of the lowest rates of all cancers.[3,4]

Similar to that of many other cancers, the molecular basis of lung cancer is genetic mutation. When mutation occurs, the expression levels of related genes and their products are altered. Genes in a cell interact with each other in pathways that regulate cell growth, division, DNA repair, and apoptosis.[5,6] Unduly expressed or under expressed genes dysregulate biochemical pathways including metabolic pathways and signaling pathways. Lung cancer is a very complex and heterogeneous disease. To develop better cancer treatments, one needs to understand the molecular mechanisms of carcinogenesis, cancer progression and metastasis and to understand how the cancer related genes interact in networks and pathways. The development of microarray technology has made it possible to describe the cellular and molecular states of cells quantitatively and to study the expression levels of many genes simultaneously.[7–10] Microarray gene expression profiling of cancer tissues and other disease tissues has been performed by many researchers.[8–16] Several groups of researchers conducted gene expression study exploring mRNA gene expression differences in normal cells and different types of lung

cancer cells including several non-small cell lung cancer cells.[14–16]

In this study, we utilized three squamous cell lung cancer microarray datasets[14–16] generated by previous researchers to identify dysregulated genes and their associated signaling pathways and to assess the involvement of hedgehog pathway in squamous cell lung cancer.

Materials and Methods

This research utilizes three microarray datasets generated by previous researches for NSCLC. The first dataset was generated by a group of researchers to identify a list of genes that can be used to classify different types of lung cancer.[14] The dataset includes the microarray gene expression profiles of 127 adenocarcinoma specimens, 21 squamous cell lung carcinomas, and 20 pulmonary cardinoids, as well as 17 normal cell samples. Affymetrix HG-U95A oligonucleotide probe microarray chips were used in the experiment. Each chip represents about 12,625 human gene probes.

The second dataset was created by another group of researchers who worked more specifically with squamous cell lung carcinomas. Their work paired normal lung tissue and squamous cell lung cancer tissue from 5 patients, and analyzed their results to find which genes were expressed differently in squamous cell cancer compared to normal cells.[15] The relations and interactions between the altered genes were also investigated. Affymetrix HG-U133A chips were used. Each chip represents 14,500 well-characterized probes.

The third dataset was produced by a group aimed at identifying highly dysregulated genes with biological significance in tumor progression.[16] The dataset has 46 tumor samples, including 13 adenocarcinomas and 32 squamous cell lung carcinomas, and 45 paired nontumor control samples. In the study, gene expression levels were investigated as a function of tumor type, stage and differentiation grade. Affymetrix HG-U133A chips were used. Each chip represents 54,675 mRNA probes.

The focus of this study was on squamous cell lung cancer. We used only the relevant subsets from the three datasets. The raw data (cel files) were provided for the first dataset, and Affymetrix's MicroArray Suite 5 (MAS5) implemented in GeneSpring software

version 11.0 (Silicon Genetics, Redwood City, CA) was used to normalize the expression levels of genes for the dataset. The 75 percentile shift was conducted for the second dataset. The median of each sample was transformed to the median of all samples for both datasets. We then carried out statistical analysis to identify the differentially expressed genes between squamous cell lung cancer cells and normal cells. The gene expression levels were log-transformed. For the first dataset, two sample Welch t-test[17] was deployed to test the null hypothesis that the mean expression level of a gene from cancer cells is the same as the mean expression level of the gene from normal cells. For the second dataset, paired t-test was used. In all tests, we assumed that samples were independently selected random samples from the two populations: squamous cell lung carcinomas tissue and normal lung tissue and the population distributions are approximately normal; this is equivalent to assume that most of the time the fluctuation of gene expression levels within cancer tissues or within normal lung tissues is not that far from the average levels and the fluctuation is symmetric to the average levels.[14–16] Multiple testing corrections based on Benjamini and Hochberg false discovery rate (FDR) were conducted.[18,19] The two-tailed P-value 0.05 was used as the cutoff for significance to obtain a set of genes that were significantly altered in squamous cell lung carcinomas. For the third dataset, the list of differentially expressed genes provided in the supplementary table to the original paper was used. They were identified using FDR correction. We then found the intersection of the three sets to obtain the genes that are commonly dysregulated in all three datasets.

The set of commonly altered genes was analyzed using the software IPA (Ingenuity® Systems, http://www.ingenuity.com). Related molecular functions and canonical pathways were identified. Top functional groups were identified using Fisher's exact test[20] with P-value 0.05 as the cutoff for significance and marked with the ratio of the number of dysregulated genes that are belong to the functional group to the total number of genes in IPA knowledge database that are annotated to the group. Significance of association between the differentially expressed genes and the canonical pathways recorded in IPA knowledge database was evaluated with the same measures, P-value from Fisher's exact test and the concentration ratio.

In addition, we examined the expression levels of genes that are associated with hedgehog (Hh) signaling pathway. Hh signaling plays important roles in a variety of normal cellular processes including embryogenesis, patterning of organ structures, tissue repair, cellular proliferation and survival.[21,22] The analysis of the connection between the altered genes with Hh pathway was performed using IPA and Fisher's exact test. P-value 0.05 was used as the cutoff.

Results and Discussion

Differentially expressed genes and their biological functions—Sorting all the P-values for the genes, we can determine which genes have the highest difference in expression levels in the samples, and therefore are most likely to have a difference in expression levels in the population. A commonly used P-value cutoff of 5% with FDR correction was used. There are 1951 genes that were significantly down regulated in the first dataset, 532 in the second dataset and 1685 genes in third dataset while 1909, 474, 1404 are up regulated respectively in the first, second and third datasets. 395 differentially expressed genes are common to all the three dataset, including 148 up-regulated and 247 down-regulated. These genes are presented in Supplemental Tables 1 and 2. The top 30 up/down regulated genes are shown in Tables 1 and 2 and the fold change of their expression levels are also presented in Figure 1. Since the experiments conducted for the three datasets were carried out in different settings using different microarray chips, in order to compare the fold change of each gene across different experiments, fold change values were normalized for each dataset.

The biological functions associated with the 395 commonly dysregulated genes were obtained using IPA, as they are most likely to be connected to the mechanism of cancer growth. The results show that the genes are associated with many molecular and cellular functions as well as different types of diseases including cancer and genetic disorder. The top molecular and cellular functions are presented in Table 3.

Canonical signaling pathways associated with differentially expressed genes—To examine dysfunctional signaling pathways, we utilized all altered genes (without FDR correction) that are common to all three datasets. FDR correction was not conducted

Table 1. Top up-regulated genes (common to all datasets).

Symbol	Fold change			Entrez gene name
	D1	D2	D3	
KRT6A	11.39	7.47	11.46	Keratin 6A
DST	5.54	5.32	5.07	Dystonin
SERPINB5	4.44	4.54	5.61	Serpin peptidase inhibitor, clade B, member 5
CLCA2	2.08	5.54	4.66	Chloride channel accessory 2
KRT17	1.91	4.55	5.82	Keratin 17
KRT15	3.56	4.84	2.64	Keratin 15
KRT16	3.20	5.08	2.51	Keratin 16
TP63	3.20	4.42	2.69	Tumor protein p63
TFAP2A	6.47	2.48	2.24	Transcription factor AP-2 alpha
AKR1B10	2.95	5.06	2.40	Aldo-keto reductase family 1, member B10
TRIM29	2.24	3.15	4.43	Tripartite motif containing 29
DSP	3.27	3.78	1.89	Desmoplakin
PKP1	1.87	4.03	2.25	Plakophilin 1
CDC20	2.41	2.94	1.92	Cell division cycle 20 homolog
GINS1	2.72	2.43	1.88	GINS complex subunit 1
UBE2C	3.05	2.13	1.86	Ubiquitin-conjugating enzyme E2C
COL17A1	2.71	2.17	1.94	Collagen, type XVII, alpha 1
CCNB1	2.00	2.88	1.86	Cyclin B1
FSCN1	1.85	2.82	1.96	Fascin homolog 1, actin-bundling protein
TPX2	2.14	2.52	1.87	TPX2, microtubule-associated
TOP2A	2.29	2.36	1.87	Topoisomerase (DNA) II alpha
IGFBP2	2.15	2.44	1.85	Insulin-like growth factor binding protein 2
AKR1C1/2	2.01	2.48	1.91	Aldo-keto reductase family 1, member C2
SOX15	2.01	2.34	1.86	SRY-box 15
SFN	1.86	2.47	1.89	Stratifin
CDK1	2.02	2.31	1.83	Cyclin-dependent kinase 1
CSTA	1.89	2.35	1.80	Cystatin A (stefin A)
CCNB2	2.17	1.98	1.85	Cyclin B2
UCK2	1.84	2.30	1.81	Uridine-cytidine kinase 2
SLC16A1	1.82	2.16	1.95	Solute carrier family 16, member 1

for identifying genes since the focus was on individual pathways.

There are 1122 differentially expressed genes with 506 commonly up-regulated and 616 down-regulated. The core analysis using IPA suggests that over 100 canonical signaling pathways are implicated in squamous cell lung cancer (Supplemental Table 3). The top altered pathways include cell cycle: G2/M DNA damage checkpoint regulation, RAR activation, Wnt/β-catenin signaling, mTOR signaling, HIF1α signaling, as well as EGF signaling. The top 40 signaling pathways implicated in squamous cell lung carcinoma are shown in Figure 2.

Downstream molecules of hedgehog signaling pathway—The hedgehog signaling pathway has been implicated in the development of different human cancers including prostate cancer, colon cancer and basal-cell carcinoma. GLI proteins are the effectors of hedgehog signaling. GLI1 and GLI2 are the two key members of GLI family and together they have 223 downstream target molecules collected in the IPA knowledge database. We analyzed the GLI target molecules for their connection with squamous cell lung cancer. Interestingly, 37 out of 223 are significantly altered. The 37 associated genes are presented in Table 4. 21 of the 37 were up-regulated. 15 of them were considerably differentially expressed in squamous cell lung cancer cells with FDR corrected P-values less than 0.05 in all three datasets (Supplemental Tables 1 and 2). Eight of them, CDC45, CDK1, CSTA, HDAC1, JAG1, JUP, KRT15, KRT17, were up-regulated; 7 of the genes, CXCL2, FHL1, FOXA2, PAPSS2, RHOB, SAP18, SYNE1, were down.

The Fisher's exact test on the 37 genes yields a P-value of 0.0484 (<0.05) which indicates the strong

Table 2. Top down-regulated genes (common to all datasets).

Symbol	Fold change			Entrez gene name
	D1	D2	D3	
CLDN18	−7.48	−4.19	−10.41	Claudin 18
ADH1B	−5.40	−7.29	−5.51	Alcohol dehydrogenase 1B, β polypeptide
AGER	−6.95	−5.17	−4.07	Advanced glycosylation end product-specific receptor
SFTPC	−5.09	−3.29	−8.35	Surfactant protein C
FABP4	−7.37	−5.13	−2.35	Fatty acid binding protein 4, adipocyte
TNNC1	−3.58	−5.02	−3.03	Troponin C type 1
CLIC5	−3.23	−5.27	−3.20	Chloride intracellular channel 5
LIMCH1	−2.81	−5.23	−3.18	LIM and calponin homology domains 1
FOLR1	−4.02	−2.40	−4.25	Folate receptor 1
PGC	−3.07	−2.64	−4.78	Progastricsin
CLEC3B	−3.07	−3.85	−3.21	C-type lectin domain family 3, member B
TCF21	−4.74	−3.91	−1.93	Transcription factor 21
FCN3	−3.90	−3.78	−2.33	Ficolin (collagen/fibrinogen domain containing) 3
HPGD	−3.24	−4.53	−2.15	Hydroxyprostaglandin dehydrogenase 15-(NAD)
ABCA8	−3.59	−4.19	−1.97	ATP-binding cassette, sub-family A, member 8
NKX2-1	−4.12	−2.28	−3.14	NK2 homeobox 1
PTPRB	−6.15	−2.17	−2.15	Protein tyrosine phosphatase, receptor type, B
CYP2B6	−2.43	−2.41	−4.75	Cytochrome P450, family 2, subfamily B, polypeptide 6
ZBTB16	−4.21	−2.69	−2.22	Zinc finger and BTB domain containing 16
FHL1	−3.29	−3.88	−1.96	Four and a half LIM domains 1
LMO3	−3.41	−1.87	−3.62	LIM domain only 3
GPM6A	−2.83	−2.95	−2.73	Glycoprotein M6 A
CHRDL1	−3.08	−3.45	−2.05	Chordin-like 1
CACNA2D2	−2.12	−2.88	−3.55	Calcium channel, voltage-dependent, alpha 2/delta subunit 2
TEK	−3.49	−3.18	−1.88	TEK tyrosine kinase, endothelial
HSD17B6	−3.10	−2.97	−2.16	Hydroxysteroid (17-β) dehydrogenase 6 homolog
FIGF	−2.41	−3.53	−2.34	c-fos induced growth factor
LPL	−2.20	−2.78	−3.11	Lipoprotein lipase
GPR116	−2.49	−3.13	−2.44	G protein-coupled receptor 116
VIPR1	−3.42	−2.32	−2.35	Vasoactive intestinal peptide receptor 1

association of hedgehog signaling with non-small cell lung cancer. The contingency table for GLI1 and GLI2 downstream molecules is given in Table 5.

Genes in a cell interact with each other through a sequence of orchestrated events in pathways. Aberrant cell signaling along the pathways breaks the organized events, promotes proliferation, inhibits apoptosis, induces angiogenesis, and facilitates the cancer to spread. Our study combined three microarray datasets to explore more stable biomarkers or drug targets for squamous cell lung cancer and the pathways implicated. The results indicate that the expression levels of many genes were altered and multiple pathways were dysfunctional in squamous cell lung carcinoma (Supplemental Tables 1 and 2, Fig. 1).

The altered genes encode cytokines, enzymes, growth factors, kinases, transporters transmembrane/ nuclear receptors and transcription regulators. They participate in variety of molecular activities in different biological processes. The molecules were located in multiple cellular locations in extracellular space, cytoplasm, nucleus, and plasma membrane.

As we can see from Table 1, the top up-regulated genes include members of the keratin family, keratin 6A, keratin 15, keratin 16, and keratin 17. Their expression levels were significantly elevated. Keratins are intermediate filament–forming proteins that provide mechanical support and perform a diversity of other functions in epithelial cells.[23–25] All four of them are epithelial keratin genes. They are associated with molecular functions such as structural constituent of cytoskeleton and protein binding and participate in cytoskeleton organization, cell differentiation, regulation of cell proliferation, epidermis
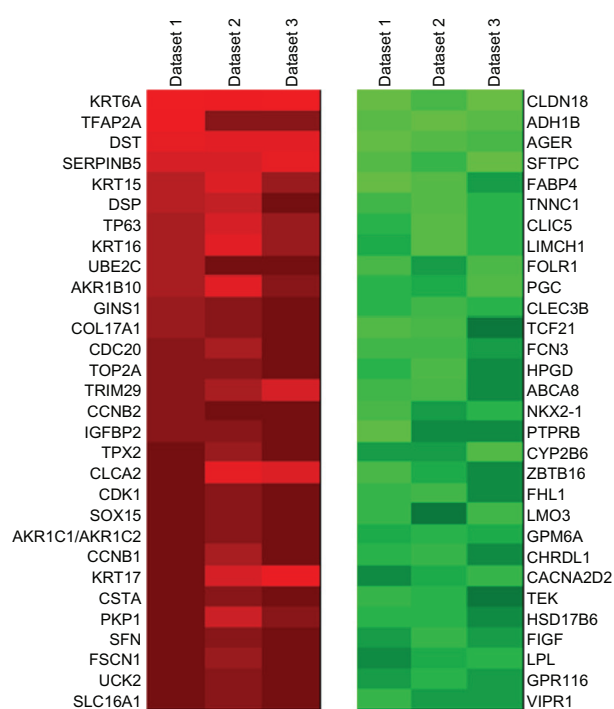
Figure 1. Heatmap showing fold change patterns of most altered genes.

development, intermediate filament cytoskeleton organization and ectoderm development.

The proteins encoded by keratin 6A are type II cytokeratins (neutral-basic). These cytokeratins organize in pairs of heterotypic keratin chains, which are co-expressed during differentiation of simple and stratified epithelial tissues. Keratin 15, 16 and 17 are acidic. Keratin 16 and 17 are paired with keratin 6 while keratin 15 is paired with keratin 5 in squamous epithelial cells. It has been reported that keratins can be used as diagnostic markers in human carcinomas.[25] A recent review on keratin summarized the evidence for active keratin involvement in cancer cell invasion and metastasis, as well as in treatment responsiveness.[25] However, their involvement in squamous cell lung carcinoma requires further investigation.

Also significantly over-expressed was cyclin-dependent kinase 1 (CDK1). The protein encoded by this CDK1 is a member of the Ser/Thr protein kinase family. It is a catalytic subunit of the highly conserved protein kinase complex known as M-phase promoting factor, which is essential for G1/S and G2/M phase transitions of eukaryotic cell cycle. The kinase activity of this protein is controlled by cyclin accumulation and destruction through the cell cycle. The phosphorylation of this protein also plays important regulatory

Table 3. Top molecular and cellular functions that are associated with commonly dysregulated genes.

Category	P-value
Cell death	(1.97E-17, 1.89E-03)
Cellular growth and proliferation	(1.13E-13, 1.29E-03)
Cell cycle	(4.01E-13, 1.79E-03)
Cellular movement	(2.88E-10, 1.93E-03)
Cellular development	(3.86E-10, 1.88E-03)
Cell-to-cell signaling and interaction	(1.01E-07, 8.62E-04)
Lipid metabolism	(3.46E-07, 1.73E-03)
Small molecule biochemistry	(3.46E-07, 1.73E-03)
Dna replication, recombination, and repair	(5.31E-06, 1.52E-03)
Gene expression	(6.49E-06, 1.79E-03)
Cellular assembly and organization	(7.95E-06, 1.15E-03)
Molecular transport	(3.89E-05, 1.73E-03)
Carbohydrate metabolism	(1.09E-04, 8.62E-04)
Protein synthesis	(1.36E-04, 5.80E-04)
Cell morphology	(1.50E-04, 1.57E-03)
Cellular function and maintenance	(3.83E-04, 1.80E-03)
Cellular compromise	(4.92E-04, 4.92E-04)
Drug metabolism	(4.92E-04, 4.92E-04)
Cell signaling	(5.80E-04, 1.14E-03)
Post-translational modification	(5.80E-04, 5.80E-04)
Antigen presentation	(8.27E-04, 8.62E-04)

roles in cell cycle control.[26–28] CDK1 is involved in many signaling pathways including apoptosis signaling; ATM signaling; cell cycle: G2/M DNA damage checkpoint regulation; cyclins and cell cycle regulation; role of CHK proteins in cell cycle checkpoint control; and sonic hedgehog signaling. In addition, several other cell-division cycle proteins including CDC20 and CDC45 were up-regulated.

The top down regulated genes are presented in Table 2. Several LIM domain proteins, FHL1, LIMCH, and LMO3, are in this group. Also included is LDB2 (Supplemental Table 2). The LIM domain is a double-zinc finger motif found in a diversity of proteins. The zinc fingers have highly conserved cysteines and joined by a 2-amino acid spacer. LIM proteins include transcription factors and cytoskeletal proteins. It is reported that the primary role of LIM domains is in protein-protein interaction, through the formation of dimers with identical or different LIM domains or by binding distinct proteins. LIM-only proteins are implicated in the control of cell proliferation.[29,30]
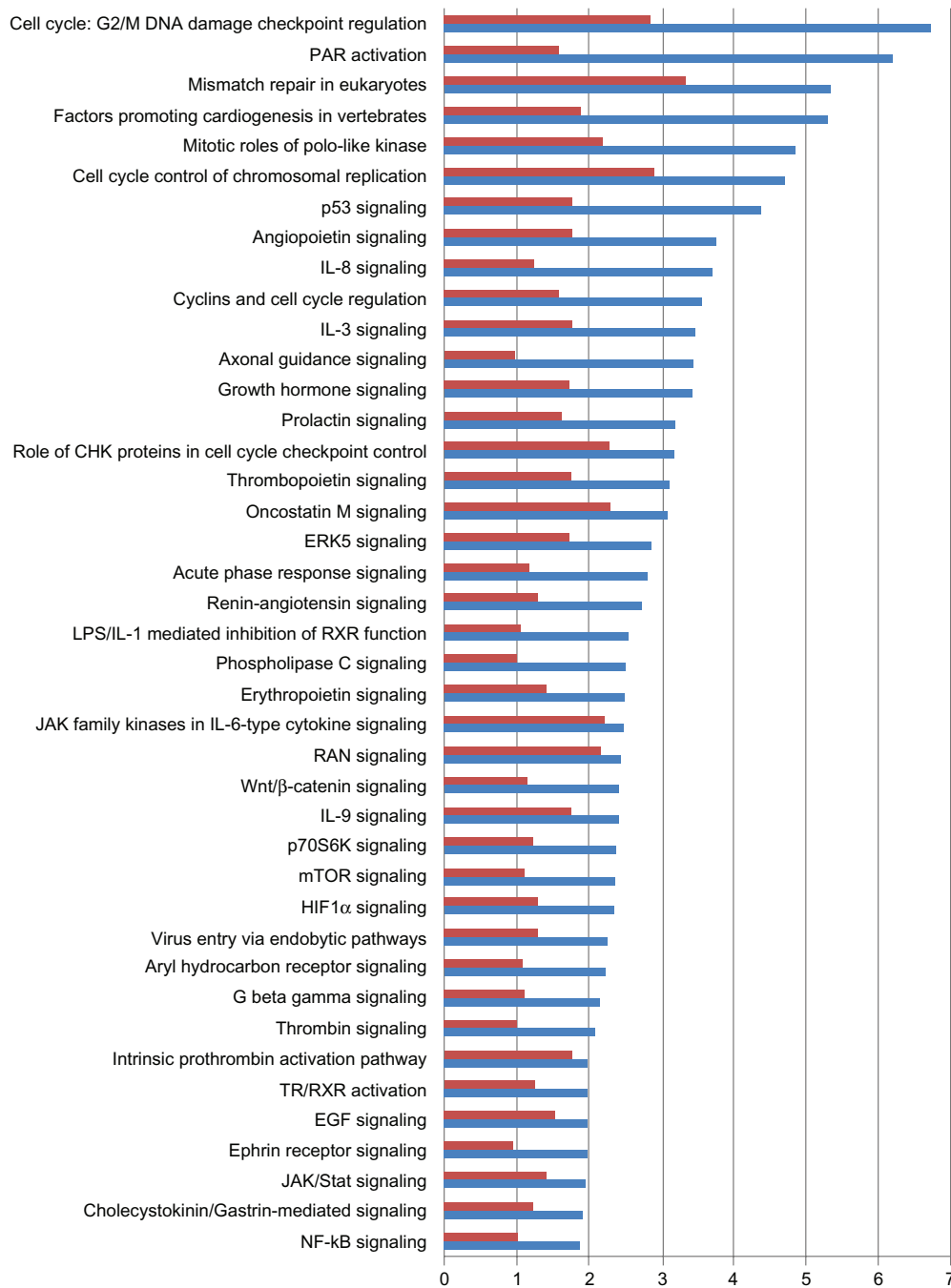
Figure 2. Altered signaling pathways linked with squamous cell lung carcinoma. The blue bars stand for –log(P-values) that were calculated based on the Fisher's exact test while the red bars represent the ratios of altered genes to the total number of genes involved in the pathways. (The red bars were scaled by a factor of 10 so that both P-values and ratios can be contrasted and presented on the same figure.).

FHL1 that encodes a member of the four-and-a-half-LIM-only protein family was significantly down regulated. Expression of these family members occurs in a cell and tissue specific mode and the proteins are involved in many cellular processes. The reduced expression of FHL1 in lung cancer has been recently reported. It was suggested that the inhibitory effects of FHL1 on lung cancer cell growth were associated with both the G1 and the G2/M cell cycle arrest associated with a marked inhibition of cyclin A, cyclin B1 and cyclin D as well as the induction of the cyclin dependent kinase inhibitors p21 and p27.[30] Conversely, several members of cyclin family (cyclin A2, cyclin B1, cyclin B2 and cyclin dependent

Table 4. GLI1/GLI2 downstream genes commonly differentially expressed in all three datasets.

Symbol	Entrez gene name	Type
CSTA↑	Cystatin A (stefin A)	Other
DIO2↑	Deiodinase, iodothyronine, type II	Enzyme
FHL1	Four and a half LIM domains 1	Other
GMFG	Glia maturation factor, gamma	Growth factor
INSIG1	Insulin induced gene 1	Other
KRT15↑	Keratin 15	Other
KRT17↑	Keratin 17	Other
PAPSS2	3′-phosphoadenosine 5′-phosphosulfate synthase 2	Enzyme
RHOB	Ras homolog gene family, B	Enzyme
SPRR2A↑	Small proline-rich protein 2A	Other
COL1A1↑	Collagen, type I, alpha 1	Other
CXCL2	Chemokine ligand 2	Cytokine
JAG1↑	Jagged 1	Growth factor
MMP9↑	Matrix metallopeptidase 9	Peptidase
MMP11↑	Matrix metallopeptidase 11	Peptidase
PI3↑	Peptidase inhibitor 3, skin-derived	Other
PTHLH↑	Parathyroid hormone-like hormone	Other
SPP1↑	Secreted phosphoprotein 1	Cytokine
TIMP3	TIMP metallopeptidase inhibitor 3	Other
BNC1↑	Basonuclin 1	Transcription regulator
CDC45↑	Cell division cycle 45 homolog	Other
CDK1↑	Cyclin-dependent kinase 1	Kinase
FOXA2	Forkhead box A2	Transcription regulator
FOXM1↑	Forkhead box M1	Transcription regulator
GATA6	GATA binding protein 6	Transcription regulator
HDAC1↑	Histone deacetylase 1	Transcription regulator
HDAC2↑	Histone deacetylase 2	Transcription regulator
HOXA5	Homeobox A5	Transcription regulator
ID2	Inhibitor of DNA binding 2	Transcription regulator
KLF4	Kruppel-like factor 4 (gut)	Transcription regulator
MEF2C	Myocyte enhancer factor 2C	Transcription regulator
SAP18	Sin3 A-associated protein, 18 kDa	Transcription regulator
SOX2↑	SRY-box 2	Transcription regulator
SYNE1	Spectrin repeat containing, nuclear envelope 1	Other
TSC22D1	TSC22 domain family, member 1	Transcription regulator
JUP↑	Junction plakoglobin	Other
PPAP2C↑	Phosphatidic acid phosphatase 2C	Phosphatase

Note: ↑Up-regulated genes.

kinase I) were found up-regulated in lung cancer in this study (Supplemental Table 1) while cyclin dependent kinase inhibitors CDKN1A and CDKN1B were consistently down-regulated in all three datasets (although the down-regulation levels were not above the statistical significant threshold).

Down-regulated RHOB is a member of the RHO GTP-binding protein family. The RHO family plays a crucial role in the dynamic regulation of the actin cytoskeleton. Studies suggested that the proteins of the family also have a diversity of functions in membrane pathways. The RHOB was shown to localize to vesicles of the endocytic compartment, suggesting a potential function in regulation of endocytic traffic. The expression of active RHOB causes a delay in the intracellular trafficking of the epidermal growth factor (EGF) receptor through regulating PRK1.[31–33]

The signaling pathway investigation in this study reveals that many of the cancer related pathways reported in the literatures are also responsible for squamous cell lung carcinoma. They include cell cycle regulation pathway ($P = 1.8E-7$), p53 tumor-suppressor pathway ($P = 4.2E-5$), IL-8 signaling ($P = 1.9E-4$), Wnt-β-catenin pathway ($P = 0.0038$),

Table 5. Contingency table for GLI1/GLI2 downstream molecules.

	GLI1/2 downstream	not GLI1/2 downstream	
Differentially expressed	37	1085	1122
Not differentially expressed	186	7573	7759
	223	8658	8881*

Note: *8881 is the total number of genes presented on the Affymetrix chips that have biological annotations in IPA.

mTOR signaling ($P = 0.0042$), and EGF signaling ($P = 0.010$) (Fig. 2).

Cell cycle: G2/M DNA damage checkpoint regulation pathway is on the top of the altered pathways. G2/M checkpoint is the second checkpoint within the cell cycle. It prevents cells from entering mitosis when DNA is damaged, providing an opportunity for repair and stopping the proliferation of damaged cells and helping to maintain genomic stability.[26] 14 out of 49 molecules on the pathway were significantly altered in lung cancer including the cyclin-dependent kinase 1 (CDK1) which is essential to drive cells into mitosis (Supplemental Tables 1 and 2).

p53 activation is induced by a number of stress signals, including DNA damage, oxidative stress and activated oncogenes. The p53 protein is employed as a transcriptional activator of p53-regulated genes. This results in cell cycle arrest, cellular senescence or apoptosis. 17 out of 49 molecules on the pathway were significantly altered, including up-regulated SERPINB5 and TP63. Serpin peptidase inhibitor, clade B (ovalbumin), member 5 (SERPINB5), also known as maspin, is a tumor suppressor gene. It was reported to block the growth, invasion, and metastatic properties of mammary tumors. Its expression was down-regulated in breast tumors, prostate cancer and uterine cervix cancer.[34–36] It was reported that SERPINB5 over-expression increased the rate of apoptosis of both preneoplastic and carcinomatous mammary epithelial cells and reduced tumor growth through a combination of reduced angiogenesis and increased apoptosis.[32] However, the role of SERPINB5 in the inhibition of tumor growth is rather complex. In contrast to its reduced expression levels in breast cancer and other types of cancer, SERPINB5's over-expression in pancreatic carcinoma was also

reported.[37] A recent study shows how it works in regulating cell growth when tightly coupled with the thrombin receptor protease activated receptor-1 (PAR-1).38 Our study indicates that SERPINB5 is significantly over-expressed in squamous cell lung carcinoma in all three datasets (Table 1). The tumor protein TP63 is another up-regulated gene (Table 1) that encodes a member of the p53 family of transcription factors. It acts as a sequence specific DNA binding transcriptional activator or repressor. Its over-expression has already been documented in lung carcinoma.[39]

EGFR encodes a receptor for members of the epidermal growth factor family. Binding of the protein to a ligand induces receptor dimerization and tyrosine autophosphorylation and leads to inappropriate activation of the anti-apoptotic Ras signaling cascade, eventually leading to uncontrolled cell proliferation. Both EGFR and the signal transducer and activator STAT1 were up-regulated in the three datasets (based on the *P*-values without FDR correction). EGF receptor signaling has long been studied in searching for the treatment of lung cancer[40,41] and EGFR inhibitors against lung cancer have been explored.[42–45] In principle, it should be feasible to regulate the activity of signaling pathways with noncytotoxic agents. Gefitinib and Erlotinib are two agents used in the treatment of non-small cell lung carcinoma. These drugs are epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, which interrupt signaling through EGFR in target cells.[44,45] They improve response rate and survival, especially in the subpopulation of patients who carry certain mutations in EGFR gene. However, clinical trials show that EGFR tyrosine kinase inhibitors , like many other drugs, have not been successful in curing cancer.[44–48]

Another important cancer related pathway is Wnt/β-catenin signaling pathway. Wnt signaling pathway is a network of proteins best known for their roles in embryogenesis and cancer, but also involved in normal physiological processes in adult animals.[46,47] Wnt proteins are secreted morphogens that are required for basic developmental processes, such as cell-fate specification, progenitor-cell proliferation and the control of asymmetric cell division, in many different species and organs.[49,50] Wnt ligand binds to its receptor and stabilizes cytoplasmic β-catenin through inhibition of the β-catenin degradation complex.

β-catenin is then free to enter the nucleus and activate Wnt-regulated genes through its interaction with TCF family transcription factors and associated recruitment of coactivators. 20 out of 174 Wnt signaling related genes are altered in the datasets. Although conflicting roles of Wnt-5 A in cancer have been reported,[51] our data shows it is significantly up-regulated and the Wnt inhibitor WIF1 is down-regulated. The *P*-values without FDR correction are consistently below the threshold in all three datasets.

mTOR pathway has been extensively explored in some other cancers, including renal cell carcinoma, and large clinical trials have reported promising progression-free survival advantage with mTOR inhibitor drugs in this disease. Our report suggests that mTOR signaling may be involved in squamous cell lung carcinoma as well, and exploring this pathway in more depth in squamous cell carcinoma of the lung may be needed.[52,53]

Many hurdles remain in the race to find a better treatment to cancer. Recently a cellular screen for antagonists of GLI-mediated transcription revealed that a small molecule GANT61 was able to selectively inhibit the GLI-mediated gene transactivation, which is the last step of the Hh pathway.[54] Hh signaling is tightly coupled with Wnt signaling.[47] It plays important roles in a variety of normal cellular processes including embryogenesis, patterning of organ structures, tissue repair, cellular proliferation and survival.[21,22] The signaling pathway is activated when hedgehog signaling ligands, such as sonic hedgehog, bind to the membrane receptor patched (PTCH), and thus stop the suppression of the signaling molecule smoothened (SMO) caused by PTCH. SMO then activates the cytoplasmic protein SUFU, which releases the GLI family of transcription factors, GLI1 and GLI2. Once the GLI molecules are released, they translocate to the nucleus, where they bind to target genes to start transcription.[21,22] The signaling process is shown in Figure 3. Hh signaling pathway is an important regulator of the embryonic development and becomes reactivated in cancer. It has been activated in several human cancers including prostate cancer, colon, and small cell lung cancer although the pathway seems to be inactive in most normal adult tissues.[55–58] The inhibition of this pathway could offer a selective way of controlling just the tumor cells. In our data, GLI2 was up-regulated
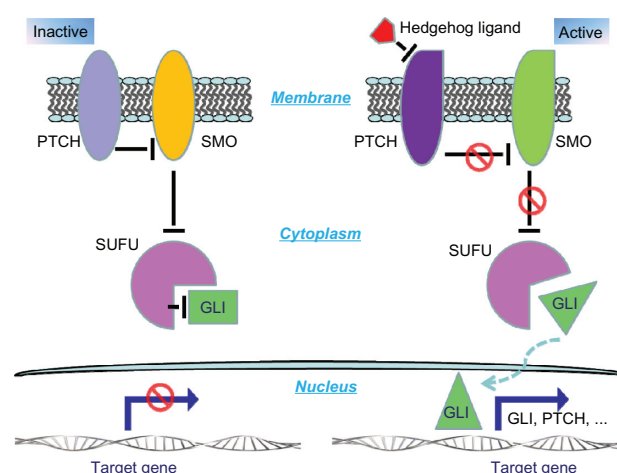

Figure 3. Mechanism of hedgehog signaling.

although the significantly altered expression level of GLI1 was not observed. On the other hand, our investigation into the group of GLI1 and GLI2 downstream molecules using IPA revealed that significant number of genes was altered in the group (Table 5). The *P*-value 0.0484 from the Fisher's exact test is below the commonly used threshold 0.05; indicating Hh signaling pathway is involved in squamous cell lung carcinoma.

Conclusion

By combining three microarray datasets, we created a more comprehensive and complete gene expression analysis of squamous cell lung cancer. The list of altered genes is more reliable than the ones obtained from a single setting of experiments, therefore providing a more dependable set of lung cancer biomarkers/drug targets. Equally the group of implicated signaling pathways is more trustworthy. The results of the analysis indicate numerous genes were altered and multiple pathways were dysfunctional in squamous cell lung carcinoma. As discussed above, many of the altered genes have been implicated in different types of carcinoma although some of the altered genes are organ-specific. Hh signaling is implicated in squamous cell lung cancer, opening the door for exploring new cancer therapeutic treatment using GLI antagonist GANT 61. While active in many different types of cancer, Hh pathway is believed to be mostly inactive in normal adult tissues, making it a good target for cancer treatments. Consequently, the treatment based on GANT 61 is likely to cause few side effects.

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Disclosures

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Supplemental Materials

Supplementary tables 1, 2 and 3 can be found in:
8283 Supplementary files.zip