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Proteomic signatures associated with p53 mutational status in lung adenocarcinoma

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Abstract

p53 is commonly mutated in lung adenocarcinoma. Mutant p53 loses wild-type function and some missense mutations further acquire oncogenic functions, while p53 wild-type may also induce pro-survival signaling. Therefore identification of signatures based on p53 mutational status has relevance to our understanding of p53 signaling pathways in cancer and identification of new therapeutic targets. To this end, we compared proteomic profiles of three cellular compartments (whole cell extract (WCE), cell surface, and media) from 28 human lung adenocarcinoma cell lines that differ based on p53 mutational status. In total, 11,598, 11,569, and 9,090 protein forms were identified in WCE, cell surface, and media, respectively. Bioinformatic analysis revealed that representative pathways associated with epithelial adhesion, immune and stromal cells, and mitochondrial function were highly significant in p53 missense mutations, p53 loss and wild-type p53 cell lines, respectively. Of note, mRNA levels of PGC1- α , a transcription co-activator that promotes mitochondrial oxidative phosphorylation and mitochondrial biogenesis, was substantially higher in p53 wild-type cell lines compared to either cell lines with p53 loss or with missense mutation. siRNA targeting PGC1- α inhibited cell proliferation in p53 wild-type cell lines, indicative of PGC1- α and its downstream molecules as potential therapeutic targets in p53 wild-type lung adenocarcinoma.

Keywords

lung adenocarcinoma; p53; PGC-1 α ; proteomics; transcriptomics

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1. INTRODUCTION

Tumor suppressor p53 plays crucial and diverse roles in physiological processes [1]. p53 is commonly mutated in human cancer [2], and a large body of evidence indicates that mutant p53 loses wild-type functions and some missense mutations further acquire oncogenic functions [3, 4]. p53 mutation is not required for tumor development as many cancers emerge with wild-type p53. Approximately 50% of lung adenocarcinomas do not harbor p53 mutation [2, 5]. In these tumors, p53 is often inactivated by two major mechanisms: One is overexpression of MDM2, which ubiquitinates p53 leading to its proteasomal degradation [6]. MDM2 is detectable by immunohistochemistry in 30%-60% of lung adenocarcinomas while its amplification occurs in less than 10% [7-10]. The other mechanism is inactivation of p14^{ARF}, which occurs in ~40% of lung adenocarcinomas, as a result of homozygous deletion, hypermethylation of promoter CpG islands, or point mutations [11]. p14^{ARF} promotes degradation of MDM2, leading to stabilization and accumulation of p53 [12]. Thus, restoration of p53 function has been an attractive strategy for cancer therapy. However recent findings have indicated that p53 wild-type in cancer cells may induce pro-survival signaling, such as induction of various genes associated with DNA repair, cell cycle regulation, oxidative stress response, and MAPK signaling, which leads to activation of anti-apoptotic pathways [13, 14]. In addition, p53 wild-type is indeed overexpressed in a subset of non-small cell lung cancers with low level expression of MDM2 protein and upregulated expression of p14^{ARF} protein [15, 16], further supporting the potential relevance of p53 wild-type to cancer cell survival.

On this basis, identification of signatures based on p53 mutational status has relevance to our understanding of p53 signaling pathways in cancer and identification of new therapeutic targets. To this end, we compared proteomic profiles of three cellular compartments from 28 human lung adenocarcinoma cell lines that differ based on p53 mutational status.

2. MATERIALS AND METHODS

Cell culture and transfection

All lung adenocarcinoma cell lines used in this study were cultured in RPMI1640 containing 10% FBS and 1% penicillin/streptomycin cocktail (Gibco). For the siRNA transfection experiments, three small interfering RNAs (siRNAs) targeting PPARGC1A (Sigma; #1: SASI_Hs01_00063323, #2: SASI_Hs01_00063324, and #3: SASI_Hs01_00063325) and negative control siRNA (Life Technologies; Cat # 4390844) were used. Cells were transfected at a final concentration of 50nM siRNA using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's instructions. 72-hours post-transfection, protein was harvested using RIPA buffer for western blotting and MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega) was performed according to the manufacturer's protocol. Three independent experiments for MTS assay were done in triplicate.

Gene expression analysis

Total RNA was extracted from lung adenocarcinoma cell lines using RNeasy Plus Mini Kit (QIAGEN). RNA quality and concentration were checked by the Experion Automated Electrophoresis System (Bio-Rad) according to manufacturer's protocol. Expression data were obtained using the Illumina Human WG-6 v3.0 Expression BeadChips (Illumina) at the Genomics Core at UT Southwestern. Bead-summarized data were obtained using the Illumina BeadStudio software, and pre-processed using the R package MBCB (Model-based Background Correction for Beadarray) for background correction and probe summarization. Pre-processed data were the quantile-normalized and log transformed.

Mass spectrometry analysis

Proteomic analysis was performed as previously described [17, 18]. All cell lines were grown in RPMI1640 (Pierce) containing 10% of dialyzed FBS (Invitrogen), 1% penicillin/streptomycin cocktail and ^{13}C -lysine instead of regular lysine, for seven passages according to the standard SILAC protocol [19]. To obtain whole cell extracts, $\sim 2 \times 10^7$ cells were lysed in 1 ml of PBS containing the detergent octyl-glucoside (OG) (1% w/v) and protease inhibitors (complete protease inhibitor cocktail, Roche Diagnostics), followed by sonication and centrifugation at $20,000 \times g$ with collection of the supernatant, and filtration through a $0.22 \mu\text{m}$ filter. The secreted proteins were obtained directly from the cell conditioned media after 48 h of culture. Cells and debris were removed by centrifugation at $5000 \times g$ and filtration through a $0.22 \mu\text{m}$ filter. To isolate cell surface proteins, $\sim 2 \times 10^8$ cells were biotinylated in the culture plate with 10 ml of 0.25 mg/ml of Sulfo-NHS-SS-BIOTIN (Thermo Scientific) in PBS at room temperature ($23\text{--}24^\circ\text{C}$) for 10 min, after extensive PBS rinsing. The residual biotinylation reagent was quenched with 10mM Lysine. Protein extraction was performed in a solution containing 2% (v/v) Igepal CA-630 (Sigma-Aldrich) with cell disruption by sonication followed by centrifugation at $20,000 \times g$. Biotinylated proteins were chromatographically isolated by affinity chromatography using 1 ml of NeutrAvidin Plus UltraLink Resin (Pierce) according to the manufacturer's instructions. Proteins bound to the column were recovered by reduction of the biotinylation reagent with 5 ml of a solution containing 65 μmol of DTT and 1% octyl-glucoside (OG) detergent for overnight at 4°C . Two mg of whole cell extracts, 1mg of conditioned media, and 0.5mg of cell surface proteins were reduced in DTT and alkylated with iodoacetamide before fractionation with reverse-phase high performance liquid chromatography (RP-HPLC). A total of 84 fractions were collected at a rate of 3 fractions/minute. The mobile phase A was: $\text{H}_2\text{O}:\text{Acetonitrile}$ (95:5, v/v) with 0.1% of trifluoroacetic acid (TFA); mobile phase B was: $\text{Acetonitrile}:\text{H}_2\text{O}$ (95:5) with 0.1% of TFA. The collected fractions from RP-HPLC were dried by lyophilization and subjected to in-solution digestion with trypsin (Trypsin Gold, Mass Spectrometry Grade, Promega). Trypsin powder was dissolved in the Digestion Buffer (100 mM amino bicarbonate, 2% Acetonitrile) to make 8ng/ μL of Trypsin solution. 50 μL of Trypsin solution was added to each of the dried fractions, capped and mixed thoroughly, followed by digestion for 5 hours at 37°C . The digestion was quenched by adding 10 μL of quench solution (1% TFA in H_2O). Based on chromatogram profile, 84 fractions were grouped into 24-pool fractions for LC-MS/MS analysis by RPLC-MS/MS using a nanoflow LC system (Eksigent) coupled on-line with Mass spectrometry (MS) Separations were performed using $75 \mu\text{m}$ inner diameter $\times$ $360 \mu\text{m}$ outer diameter $\times$ 25cm long fused silica

capillary column (New Objective) slurry packed in house with 5 μ m, 200Å pore size C18 silica-bonded stationary phase (Magic C18 AQ, New Objective). Following injection of ~2 μ g of protein digest onto a C18 trap column (Waters, 180 μ mID $\times$ 20mmL), the LC column was washed for 5min with mobile phase A (2% acetonitrile, 0.1% formic acid) at a flow rate of 10 μ L/min. Peptides were eluted using a linear gradient of 0.35% mobile phase B (0.1 formic acid in acetonitrile)/minute for 90 min, then to 95% B in an additional 10 min, all at a constant flow rate of 300nL/min. Eluted peptides were analyzed by MS using an LTQ Orbitrap mass spectrometer (Thermo Scientific) in data dependent acquisition (DDA) mode. Each full MS scan (m/z 400-1800) was followed by 10 MS/MS scans (normalized collision energy of 35%) for the 10 most abundant precursor ions in a ~1.5s of duty cycle. Dynamic exclusion was enabled to minimize redundant selection of peptides previously selected for MS/MS analysis. Parameters for MS1 were 60,000 for resolution, 1×10^6 for automatic gain control (AGC) target, and 150 ms for maximum injection time. MS/MS was done by collision-induced dissociation (CID) fragmentation with 3×10^4 for AGC, 10 ms for maximum injection time, 35 for normalized collision energy (NCE), 2.0 m/z for isolation width, 0.25 for activation q -value, and 10 ms for activation time. MS/MS spectra were searched against the IPI proteome database (IPI human, v3.57 and IPI bovine, v3.43) using SEQUEST through the Computational Proteomics Analysis System, CPAS (LabKey Software Foundation, <https://www.labkey.org/Project/home/CPAS/begin.view>) with one fixed modification of carbamidomethylation at Cysteine (57.04304 Da) and two variable modifications, oxidation at Methionine (15.9949 Da) and SILAC at Lysine (6.0201 Da). Peptides were considered legitimately identified if they achieved specific charge state and proteolytic cleavage-dependent cross-correlation (Xcorr) scores of 1.9 for $[M+H]^+$, 2.2 FOR $[M+2H]^{2+}$, and 3.5 for $[M+3H]^{3+}$, and a minimum delta correlation score (Cn) of 0.08. A false protein discovery rate of approximately 5% was determined by searching the primary tandem MS data using the same criteria against a decoy database wherein the protein sequences are reversed. To eliminate the possible contamination of bovine proteins, any unlabeled lysine containing peptides with bovine homology were discarded. The total number of spectral counts for each protein group was normalized to total spectral counts of the samples which were set as 50,000, and used for the differential analysis among different samples. Total spectral counts were normalized to 50,000 total events for each cell line.

Western blot analysis

Anti-PGC-1 α antibodies were obtained from Cell Signaling Technology used for Western blot analysis. Anti-GAPDH antibody (Abcam) and β -tubulin (Cell Signaling Technology) were used as loading control. Signal intensities of PGC-1 α protein bands were quantified using ImageJ (<http://rsbweb.nih.gov/ij/>) and normalized to β -tubulin signal intensities.

3. RESULTS

Proteomic profiling of lung adenocarcinoma cell lines

Three cellular protein compartments (whole cell extract (WCE), cell surface, and media) of 28 human lung adenocarcinoma cell lines, including 23 with mutant p53 and 5 with wild type p53, were analyzed by mass spectrometry (Supporting Information Tables 1, 2, and 3). p53-mutant cell lines were further classified into two groups: 1- with missense mutation and

2- with p53-loss, as p53 missense mutations have the potential to gain oncogenic functions (Table 1) [3, 4]. p53 mutational status was not associated with phenotypic characteristics such as cell morphology or invasion propensity nor with the mutational status of EGFR or KRAS, which are major oncogenic drivers in lung adenocarcinoma. In total, 11,598, 11,569, and 9,090 protein forms were identified in WCE, cell surface, and media, respectively. Average number of identified proteins in each compartment (Mean $\pm$ Standard Deviation (SD)) was 3380.7 ± 726.1 in WCE, $3,291.4 \pm 694.3$ in surface, and $1,987 \pm 451.3$ in media (Table 1). Next we assessed the enrichment of membrane proteins in the cell surface compartment and of extracellular proteins in the media compartment. Localization of proteins was predicted using the Ingenuity Pathways Analysis (IPA; <http://www.ingenuity.com/>) and protein abundance was estimated based on normalized MS/MS counts [17]. The subcellular localization demonstrated by mass spectrometric analysis and predictions from database analysis were concordant, particularly for abundant proteins (Supporting Information Figure 1), indicating enrichment of cell surface or extracellular proteins based on cellular compartment. Interestingly, cytoplasmic proteins that lack a signal peptide were also enriched in the conditioned media, suggesting potential release of cytoplasmic proteins via extracellular vesicles in these lung adenocarcinoma cells [20].

Proteomic characterization of lung adenocarcinoma cell lines based on p53 mutational status

Protein abundance based on normalized MS/MS counts was compared among 19 cell lines with p53 missense mutations, four cell lines with p53 loss, and five p53 wild-type cell lines. The following criteria were used: (1) a protein is identified in 50% or more cell lines in the group, (2) ratio of normalized MS/MS counts between two groups > 2 , and (3) average of MS/MS counts in the group > 2 . A total of 118, 66, and 112 proteins were found to be increased in WCE, cell surface, and media of cell lines with missense mutations compared to cell lines with p53 loss and p53 wild-type cell lines, respectively (Supporting Information Figure 2A). Similarly, 289 (WCE), 338 (surface), and 351 (media) proteins were elevated in cell lines with p53 loss, and 167 (WCE), 180 (surface), and 82 (media) proteins were elevated in p53 wild-type cell lines (Supporting Information Figure 2B and C). Next, we performed pathway analysis of these elevated proteins using IPA. Canonical pathways associated with epithelial adhesion (Epithelial Adherens Junction Signaling and Remodeling of Epithelial Adherens Junction) were highly significant in all three compartments of cell lines with p53 missense mutations (Figure 1A). These two pathways include 13 proteins (ARPC1B, ARPC2, ARPC5L, CDH1, CTNND1, JUP, LMO7, MET, PARD3, RAB7A, RAP1B, TGFB2, TUBB4B) (Supporting Information Table 4). Among them, protein expression levels of TGFB2, RAB7A and LMO7 in media and ARPC5L in WCE were significantly elevated in cell lines with p53 missense mutations ($P < 0.05$, Mann-Whitney U test) (Figure 2A). For cell lines with p53 loss, pathways associated with immune and stromal cells (Antiproliferative Role of TOB in T Cell Signaling, T Helper Cell Differentiation Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis Interestingly) were highly significant in WCE and cell surface (Figure 1B). CALML5, CD40, CDKN1B, CSF1, CUL1, IL18, LRP1, MMP1, NGFR, PRSS1, PRSS3, RAC1, ROR2, SKP1, STAT4, and TGFB1 were included in these pathways and protein expression levels of LRP1, MMP1 and ROR2 in the cell surface compartment were significantly elevated in cell lines with p53

loss ($P < 0.05$, Mann–Whitney U test) (Figure 2B). Four of five top significant canonical pathways in TCE of p53 wild-type cell lines were involved in mitochondrial function: Oxidative Phosphorylation, TCA Cycle II (Eukaryotic), Branched-chain α -keto acid Dehydrogenase Complex, and Mitochondrial Dysfunction (Figure 1C). Of note, p53 was predicted as one of the top significantly activated upstream regulators in all three cellular compartments in p53 wild-type cell lines, suggesting functional roles of p53 signaling pathways in p53 wild-type lung adenocarcinoma cell lines (Table 2). To further investigate key molecules associated with p53 wild-type signatures, we compared mRNA expression among the 28 lung adenocarcinoma cell lines profiled. Fifty-one probes were elevated in p53 wild-type cell lines compared to cell lines with p53 loss according to the following criteria: ratio of $\log_2$ intensity of wild-type vs p53 loss > 2 with P value of less than 0.01 (unpaired t test). Similarly, mRNA expression levels of 109 probes were significantly higher in p53 wild-type cell lines compared to cell lines with p53 missense mutations (Figure 3A).

We found that the mRNA expression level of PPARGC1A is substantially higher in p53 wild-type cell lines compared to either cell lines with p53 loss ($P = 0.0035$, unpaired t test) or with missense mutation ($P = 0.0003$, unpaired t test) (Figure 3B). PGC1- α , encoded by PPARGC1A, is a transcription co-activator that promotes mitochondrial oxidative phosphorylation and mitochondrial biogenesis [21], thus suggestive of PGC1- α as a key molecule associated with p53 wild-type function. Protein expression of PGC1- α was significantly associated with PPARGC1A mRNA expression levels in 10 lung adenocarcinoma cell lines (Spearman $r = 0.81$, $P = 0.0072$) (Supporting Information Figure 3A-C). We therefore investigated the effect of suppressing PGC1- α . Indeed, cell proliferation was inhibited with treatment of PGC1- α siRNA in H1944 cells (Fig. 3C and D), indicating PGC1- α as a potential therapeutic target in p53 wild-type lung adenocarcinoma. We also sought to identify PGC1- α -associated proteins in p53 wild-type cell lines. Based on IPA, 368 proteins have either direct or indirect interaction with PGC1- α . Among elevated proteins in p53 wild-type cell lines determined as above (Supporting Information Figure 2C), 19, 16, and 9 proteins were PGC1- α -interacting proteins in WCE, cell surface, and media of p53 wild-type cell lines respectively (Supporting Information Table 5).

4. DISCUSSION

In this study, we profiled the proteome and transcriptome of 28 human lung adenocarcinoma cell lines to identify inherent signatures associated with p53 mutational status. Protein signatures associated with p53 missense mutations were comprised of proteins involved in epithelial adhesion. Interestingly, this includes a general trend for higher surface localization of the epithelial marker CDH1 and the proto-oncogene MET. Detection of CDH1 indicates an epithelial phenotype in the context of epithelial-to-mesenchymal transition (EMT) [22], while activation of the MET pathway by HGF is known to induce EMT leading to the suppression of CDH1 [23]. Missense p53 mutations can induce EMT by several mechanisms such as influencing TGF β pathway signaling or modulating ZEB1 expression. TGF β 2 was indeed significantly elevated in the media of cell lines containing missense p53 mutations. Recently, it has been reported that TGFB2 is upregulated by mutant p53 through its gain-of-function ability to bind to and sequester p63, which indirectly represses several genes

associated with EMT, including TGF β 2 [24]. TGF β pathway activation induces EMT in a variety of cell lines [22, 25]. TGF β signaling requires the binding of TGF β first to the type I and II TGF β receptor (TGFBR2) [25]. Expression levels of TGFBR1 and TGFBR2 were very low in the missense p53 cell lines profiled (data not shown), suggesting that TGF β 2 secreted in the media is not sufficient to induce EMT in these cell lines. Missense p53 mutations can also potentiate EMT induction by MET by regulating its expression and/or enhancing MET cell surface recycling [3, 4]. The detection of both CDH1 and MET on the surface, however, suggests that these cells are poised to engage EMT driven by MET, but retain an epithelial phenotype in the absence of autocrine HGF stimulation as HGF was not identified in the secretome from lung adenocarcinoma cell lines profiled in this study (data not shown). Therefore additional molecular programs might be required to induce full EMT in these cell lines.

For cell lines with p53 loss, ROR2 is of particular interest as it is a receptor type tyrosine kinase. ROR2 regulates Wnt signaling pathways, and overexpression and oncogenic functions of ROR2 has been reported in various types of cancer [26-31]. ROR2 was highly expressed in H2291 cell line which harbors p53 missense mutation. However levels of basal p53 levels were very low in H2201 cell lines (data not shown), so p53 G154V mutation occurred in H2291 cells might be loss of function mutation.

Proteomic signatures associated with p53 wild-type are associated with mitochondrial function. p53 regulates various metabolic pathways including mitochondrial respiration [32, 33]. We found that the mRNA expression level of PPARGC1A, which encodes PGC-1 α , is substantially higher in p53 wild-type cell lines compared to either cell lines with p53 loss or with missense mutation. Although PGC-1 α is a known transcriptional target of p53, it is context-dependent whether PGC-1 α would be induced or repressed [34, 35]. The main physiological functions of PGC-1 α is control of energy metabolism by increasing oxidative metabolism, and in particular mitochondrial oxidative phosphorylation (OxPhos) in muscle, brain, liver and adipose tissue and also inhibit apoptosis [21]. Although the role of PGC-1 α in cancer is still controversial, recent papers have indicated the oncogenic roles of PGC-1 α in cancer [21, 36-39]. In addition PGC-1 α activates proarrest and metabolic target genes by modulating p53 transactivation function and promote cell survival under metabolic stress [40]. Furthermore, mitochondrial function is required for transformation and tumor growth [41, 42]. Therefore p53-PGC-1 α axis would be relevant to induce cancer-associated metabolic changes in p53 wild-type lung adenocarcinoma. Therapeutic targets for p53 wild-type cancer have not been well studied: miR-197 has been identified as a potential therapeutic target in p53 wild-type non-small cell lung cancer [43], and Nutlin-3, a MDM2 antagonist, preferentially sensitizes p53 wild-type cancer to Death Receptor 5-induced apoptosis in p53 wild-type cancer [44]. Unfortunately, PGC-1 α itself is not currently druggable [21]. Thus identification of key transcriptional partners or downstream molecules of PGC-1 α would enable us to inhibit specific pathways regulated by PGC-1 α in cancer. Bioinformatic analysis of our proteomic profiling identified many potential therapeutic targets which are associated with PGC-1 α (Supporting Information Table 5), such as SOD1 and SOD2. SOD inhibitor, 2-methoxyestradiol, is currently undergoing clinical trials for treatment of a variety of cancers [45-47].

In conclusion, proteomic characterization of lung adenocarcinoma cell lines based on p53 mutational status, integrating transcriptome and bioinformatic analysis, identified molecular pathways and potential therapeutic targets associated with p53 missense mutations, p53 loss and wild-type p53.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
WCE	whole cell extract
IPA	Ingenuity Pathways Analysis
EMT	epithelial-to-mesenchymal transition

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Figure 1. Top 5 significant canonical pathways for elevated proteins in three cellular compartments predicted by Ingenuity Pathway Analysis

Top 5 significant canonical pathways for cell lines with p53 missense mutation (A), cell lines with p53 loss (B), and p53 wild-type cell lines (C). P values were calculated by Fisher's exact test.

Figure 2. Signature proteins significantly elevated in mutant p53 lung adenocarcinoma cell lines
 A. Protein expression levels of ARPC5L in WCE, and LMO7, RAB7A, and TGFB2 in media. B. Protein expression levels of LRP1, MMP1 and ROR2 in cell surface. P values were calculated by Mann–Whitney U test.

Figure 3. PGC-1α as a potential target of p53 wild-type lung adenocarcinoma

A. Venn diagrams of genes elevated in p53 wild-type lung adenocarcinoma cell lines. B. mRNA expression of PPARGC1A in lung adenocarcinoma cell lines. Bars indicate mean and standard deviation of log₂ intensity. P value was calculated by unpaired t test. C. Knockdown of PGC1-α with siRNA in H1944 cells. GAPDH was served as a loading control. D. MTS assay of H1944 cells treated with negative control siRNA or siRNAs against PGC1-α. Columns indicate the average of triplicate samples from a representative experiment, and bars indicate standard deviation.

Table 1

Characteristic of lung adenocarcinoma cell lines and number of identified proteins.

Group	Cell line	Characteristics				Mutational Status				Number of identified proteins			
		Age	Gender	Site	Smoking	EGFR	Kras	Mutation		WCE	Surface	Media	
Missense	H1355	53	M	M	Y	Wild	Mutant	E285K		3364	3275	1973	
	H1373	56	M	NA	Y	Wild	Mutant	P47L		3380	3557	2147	
	H1437	60	M	M	Y	Wild	Wild	R267P		4087	3063	1965	
	H1573	35	F	M	N	Wild	Mutant	R248L		3282	3076	2405	
	H1651	71	M	P	NA	Wild	Wild	C176Y		3495	3837	2378	
	H1793	52	F	P	NA	Wild	Wild	R209*, R273H		3286	4267	2058	
	H1838	NA	F	P	NA	Wild	Wild	R273L		3330	3571	1679	
	H1975	NA	F	P	N	Mutant	Wild	R273H		2715	3128	1150	
	H1993	47	F	M	Y	Wild	Wild	C242W		3734	1842	1669	
	H2009	68	F	M	Y	Wild	Mutant	R273L		2143	2766	1927	
	H2030	NA	M	M	N	Wild	Mutant	G262V		2787	3063	1395	
	H2122	46	F	M	Y	Wild	Mutant	Q16L, C176F		4358	4165	2193	
	H2291	NA	M	M	N	Wild	Mutant	G154V		4360	3958	2333	
	H23	51	M	P	Y	Wild	Mutant	M246I		1463	1880	1390	
	H2342	55	M	P	NA	Wild	Wild	Y220C		4095	4060	2008	
	H2405	47	M	M	Y	Wild	Wild	R273H		3724	3441	2632	
	H650	NA	M	M	N	Wild	Mutant	K164N		3310	2692	1884	
	H820	53	M	M	NA	Mutant	Wild	E284K		4517	3894	3179	
	HCC2279	NA	F	NA	N	Mutant	Wild	Y234C		3446	3259	2311	
Loss	H2228	NA	F	P	N	Wild	Wild	Q331*		3475	3324	2088	
	H838	59	M	M	Y	Wild	Wild	E62*		2147	2502	2034	
	H522	60	M	P	Y	Wild	Wild	P191fs*56		4072	4200	2745	
	H1650	27	M	M	Y	Mutant	Wild	c.673-2A>G; Splicing Frameshift		3580	3242	1739	
	H1385	49	F	M	Y	Wild	Mutant	None		4187	4100	1769	
Wild	H1395	55	F	P	Y	Wild	Wild	None		3645	3719	1548	
	H1563	NA	M	P	NA	Wild	Wild	None		2800	3442	1986	

Group	Cell line	Characteristics				Mutational Status			Number of identified proteins			
		Age	Gender	Site	Smoking	EGFR	Kras	Mutation	WCE	Surface	Media	
	H1568	NA	F	M	Y	Wild	Wild	None	3555	3185	1933	
	H1944	NA	F	M	Y	Wild	Mutant	None	2323	1652	1133	
								Total	11598	11569	9090	
								Mean	3380.7	3291.4	1987.5	
								SD	726.1	694.3	451.3	

Table 2

Top 5 upstream regulators predicted by Ingenuity Pathway Analysis.

	WCE			Surface			Media		
	Upstream Regulator	Activation z-score	p-value of overlap	Upstream Regulator	Activation z-score	p-value of overlap	Upstream Regulator	Activation z-score	p-value of overlap
Missense	GLI1	2.646	4.56E-05	miR-16-5p	-1.982	2.02E-03	IL6	2.881	5.84E-03
	Vegf	2.630	5.20E-03	RAF1	1.980	2.55E-03	NFE2L2	2.797	6.59E-04
	PPARG	2.570	4.49E-03	HGF	1.956	1.07E-02	IL1B	2.729	5.44E-03
	MGEA5	-2.449	1.05E-03	OSM	1.531	1.37E-02	miR-30c-5p	-2.630	9.81E-07
	miR-124-3p	-2.449	1.08E-03	HNF4A	1.114	1.68E-02	IFNG	2.626	2.76E-03
Loss	NFE2L2	3.132	1.66E-02	TNF	4.453	2.93E-06	TP53	3.159	1.64E-05
	miR-16-5p	-2.800	2.17E-03	NFKB (complex)	3.804	8.51E-04	miR-16-5p	-2.985	1.85E-03
	TP73	2.596	1.46E-02	IFNG	3.602	7.09E-06	ANGPT2	2.813	1.50E-03
	CD40LG	2.596	6.06E-03	Vegf	3.410	1.59E-03	miR-1	-2.772	7.42E-03
	HIF1A	2.558	4.96E-03	FOXO1	3.286	1.41E-03	TP73	2.763	1.43E-05
Wild	TP53	3.417	5.00E-11	IFNG	3.092	8.90E-03	TP53	3.340	5.95E-07
	INSR	2.596	2.84E-06	NFE2L2	3.082	5.99E-04	FGF2	2.599	1.13E-04
	IL2	2.581	6.92E-03	TNF	3.026	1.68E-02	Vegf	2.404	1.98E-03
	IL4	2.577	2.72E-02	TP53	2.879	3.02E-10	HRAS	2.177	4.02E-04
	Map4k4	-2.449	1.30E-04	TGFB1	2.825	5.32E-06	CEBPA	2.158	2.47E-05