Altered expression of cellular proliferation, apoptosis and the cell cycle-related genes in lung cancer cells with acquired resistance to EGFR tyrosine kinase inhibitors

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Abstract. Non-small cell lung cancers harboring somatic gain-of-function mutations in the epidermal growth factor receptor (EGFR) tyrosine kinase domain respond well to treatment with EGFR tyrosine kinase inhibitors (TKIs) including gefitinib and erlotinib. However, all patients who experience a marked improvement with these drugs eventually develop disease progression due to the acquisition of drug resistance. Approximately half of the cases with acquired resistance to EGFR TKIs can be accounted for by a second-site mutation in exon 20 of the EGFR kinase domain (T790M). However, the changes of gene expression involved in EGFR TKI resistance due to the T790M mutation remain poorly defined. The present study established lung cancer cell lines that were resistant to gefitinib or erlotinib, and these cell lines were verified to contain the *EGFR* T790M mutation. The differential expression of genes associated with acquired resistance was verified in the present study by mRNA microarray analysis. Among the genes whose expression was significantly altered, genes whose expression was altered in gefitinib- and erlotinib-resistant cells were focused on. Notably, a total of 1,617 genes were identified as being differentially expressed in gefitinib- and erlotinib-resistant cells. Indeed, Gene ontology analysis revealed altered expression of genes involved in the regulation of cellular proliferation, apoptosis, and the cell cycle in EGFR TKI-resistant cells. The present results demonstrate distinctive gene expression patterns of EGFR TKI-resistant lung cancer cells with the *EGFR* T790M mutation. The present study can provide key insights into gene expression profiles involved in conferring resistance to EGFR TKI therapy in lung cancer cells.

Introduction

The epidermal growth factor receptor (EGFR) signaling pathway is importantly implicated in tumor cell growth, invasion, angiogenesis and metastasis (1). Molecular aberrations on the EGFR pathway are the most commonly studied predictive biomarkers of response to targeted agents in lung cancer. Non-small cell lung cancers (NSCLCs) harboring somatic gain-of-function mutations in the EGFR tyrosine kinase domain respond well to treatment with the EGFR tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib (2,3). These small molecule TKIs compete with ATP to bind the kinase domain of their targets. Several distinct activating mutations of the *EGFR* gene have been described in NSCLC, including in-frame deletions in exon 19 (delE746-A750) and a leucine-to-arginine substitution at position 858 (L858R) in exon 21 (2,3).

All patients who experience a marked improvement with these drugs eventually develop progression of disease subsequent to a median of 12 months due to the acquisition of drug resistance (4). Approximately half of the cases with acquired resistance to EGFR TKIs can be accounted for by a second-site mutation in exon 20 of the EGFR kinase domain, which results in the substitution of methionine for threonine at position 790 (T790M) (5,6). The bulkier methionine residue at position 790 sterically hinders binding of either gefitinib or erlotinib to the ATP-binding pocket. An additional study has indicated that T790M also increases the affinity of EGFR for ATP, thereby out-competing ATP-competitive TKIs and restoring enzymatic activity in their presence (7). However, the changes of gene expression involved in EGFR TKI resistance due to the T790M mutation remain poorly defined. Amplification of *MET*, a gene encoding a different membrane bound receptor tyrosine kinase, is a separate mechanism of acquired resistance to EGFR TKIs (8). Less frequent forms of acquired resistance include histological transformation to small cell lung cancer (9,10), *PIK3CA* mutation (9,11) and epithelial to

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mesenchymal transition (9,12). However, the exact frequencies of these mechanisms remain to be established.

Previously, gene expression profiling of human cancers has proved valuable in studies into cancer, providing insights into mechanisms and targets involved in carcinogenesis and drug response in several different types of cancer (13,14). Analysis using mRNA microarrays allows simultaneous assessment of the expression of thousands of genes and this approach provides a valuable means to identify novel molecular targets for therapeutic intervention. Additionally, it may be used to identify genes whose expression is changed in cells with acquired drug resistance by comparing gene expression in drug-resistant cells to that in parental cells that are sensitive to treatment with, for example, EGFR TKIs.

To additionally investigate resistance to EGFR TKIs, the present study has established cell lines that are resistant to either gefitinib or erlotinib. Using mRNA microarrays, genome-wide analysis of gene expression profiles has established a clear division between parental and resistant cells, with altered expression of genes involved in the regulation of cellular proliferation, apoptosis and the cell cycle in the EGFR TKI-resistant cells. The present study may provide key insights into gene expression profiles involved in conferring resistance to EGFR TKI therapy in lung cancer cells.

Materials and methods

Cell culture and establishment of the gefitinib- and erlotinib-resistant cell lines. The *EGFR*-mutated NSCLC cell line, PC-9, was used. PC-9 cells are known to contain a deletion in exon 19 (delE746-A750) of *EGFR* and be highly sensitive to gefitinib and erlotinib (15). Gefitinib- or erlotinib-resistant sublines of PC-9 were established as described previously (16) and these resistant cells were >100 times more resistant to gefitinib and erlotinib compared with parental cells. Briefly, parental PC-9 cells were exposed to 10 nmol/l of gefitinib or erlotinib for 48 h in medium containing 10% fetal bovine serum. Subsequently, cells were washed and cultured in drug-free medium until surviving cells were 80% confluent. These cells were then re-exposed to increasing concentrations of gefitinib or erlotinib. Six months after initial exposure, cells were able to grow in 1 μmol/l of gefitinib or erlotinib. The established drug-resistant cell lines were then maintained in medium containing 1 μmol/l of gefitinib or erlotinib. Gefitinib- or erlotinib-resistant cells are referred to as PC-9G and PC-9E, respectively. PC-9G and PC-9E cells were confirmed to contain the *EGFR* T790M mutation by DNA sequencing (16). All cells were maintained as monolayer cultures in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1% sodium pyruvate at 37°C in a humidified incubator in an atmosphere of 5% CO_2. All cell culture materials were obtained from HyClone (GE Healthcare, Logan, UT, USA).

mRNA microarray analysis. Duplicated total RNA sample of each cell lines was prepared using TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. The quantity and integrity of extracted RNA was confirmed using a 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) prior

to samples being deemed suitable for microarray analysis. Fluorescent complimentary RNA (cRNA) probes were generated and purified using the Agilent's Low RNA Input Linear Amplification kit (Agilent Technologies, Inc.) according to the manufacturer's protocol. Labeled cRNA target was quantified using a NanoDrop-1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). mRNA microarray hybridization was performed for the samples using the Agilent Whole Human Genome Oligo Microarray (44 K) according to manufacturer's protocol. Briefly, 600 ng Cy3-labeled fragmented cRNA was hybridized overnight to an Agilent Whole Human Genome Oligo Microarray, washed twice, blocked, and scanned using Agilent's microarray scanner.

Data acquisition and statistical analysis. The hybridized images were quantified using the Feature Extraction Software (Agilent Technologies, Inc.). All data normalization and the selection of fold-changed genes were performed using the GeneSpring GX 7.3 (Agilent Technologies, Inc.). The averages of normalized ratios were calculated by dividing the average of normalized signal channel intensity by the average of normalized control channel intensity. Functional annotation of genes was performed according to the Gene Ontology™ Consortium (http://www.geneontology.org/index.shtml) using the GeneSpring GX 7.3. Gene functional classification was based on searches carried out using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatics resources (http://david.abcc.ncifcrf.gov/).

Results

Distinctive gene expression patterns are revealed by mRNA microarray analysis in EGFR TKI-resistant lung cancer cells with the EGFR T790M mutation. To detect expression changes in the EGFR TKI-resistant cells, an mRNA microarray analysis of PC-9G and PC-9E cells in comparison with drug-naïve parental PC-9 cells was performed. In total, >20,000 genes were reliably analyzed following quantile normalization, and those showing fold changes of >1.5 were defined as being differentially expressed between the parental and either the PC-9G or PC-9E cells. Among the genes whose expression was significantly altered, the present study focused on those genes whose expression was altered in PC-9G and PC-9E cells (Fig. 1A). A total of 1,617 genes were identified as being differentially expressed in PC-9G and PC-9E cells. Cluster analysis of these differentially expressed genes between PC-9G/PC-9E and parental PC-9 cells identified 451 mRNAs with significantly increased expression in PC-9G and PC-9E compared with in PC-9 cells, and 1,166 mRNAs with significantly reduced expression in PC-9G and PC-9E compared with in PC-9 cells.

Gene ontology analyses of microarray data reveals functional pathways associated with altered mRNA expression in PC-9G and PC-9E cells. Genes were functionally categorized with regard to their associated biological process. Gene ontology analysis for the >1.5-fold up- or downregulated genes in PC-9G and PC-9E cells was performed using the DAVID tools. According to the DAVID gene ontology program, 1,104 (68%) of the 1,617 genes are associated with a known annotated

Table I. Altered expression of genes involved in cellular proliferation and apoptosis.

			Fold change (vs. PC-9)	
Gene symbol	Description	Genbank	PC-9G	PC-9E
Upregulated				
ARHGEF2	Rho/Rac guanine nucleotide exchange factor (GEF) 2	NM_004723	1.52	2.29
INPP5D	Inositol polyphosphate-5-phosphatase, 145 kDa	NM_001017915	3.49	5.22
Downregulated				
ADORA1	Adenosine A1 receptor	NM_000674	-2.90	-5.15
CD24	CD24 molecule	NM_013230	-1.91	-4.39
CLU	Clusterin	NM_001831	-3.24	-1.79
EGLN3	Egl-9 family hypoxia-inducible factor 3	NM_022073	-1.59	-2.18
F2R	Coagulation factor II (thrombin) receptor	BC016059	-5.15	-1.68
IL6	Interleukin 6 (interferon, β2)	NM_000600	-1.65	-1.49
MAGED1	Melanoma antigen family D, 1	NM_001005333	-1.86	-2.08
NTN1	Netrin 1	NM_004822	-1.63	-2.68
PHB	Prohibitin	NM_002634	-2.07	-1.58
PRKRA	Protein kinase, interferon-inducible double stranded RNA dependent activator	NM_003690	-2.74	-1.43
SMO	Smoothened frizzled class receptor	NM_005631	-3.72	-10.07
TGM2	Transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	NM_198951	-1.81	-2.02
TNFRSF14	Tumor necrosis factor receptor superfamily, member 14	AB208808	-1.76	-2.05
TNFRSF8	Tumor necrosis factor receptor superfamily, member 8	NM_001243	-2.78	-2.27

biological process, 288 (64%) of the 451 upregulated genes and 816 (70%) of the 1,166 downregulated genes. In particular, 96, 115 and 44 genes are associated with the regulation of cellular proliferation, apoptosis and the cell cycle, respectively (Fig. 1B).

Altered expression of genes involved in the regulation of cellular proliferation, apoptosis and the cell cycle. The present study subsequently investigated the genes whose expression was significantly altered in PC-9G and PC-9E cells and that were common in any two or all three ontological clusters: Regulation of cellular proliferation, apoptosis and the cell cycle (Fig. 2A). In total, 40 genes are involved in any two or all three ontological clusters. With the exception of genes involved in all of three ontological clusters, 16 genes are involved in cellular proliferation and apoptosis (Fig. 2B, Table I), six in apoptosis and the cell cycle (Fig. 2B, Table II), and five in the cell cycle and cellular proliferation (Fig. 2B, Table III). A total of 13 genes were identified to be involved in cellular proliferation, apoptosis and the cell cycle (Fig. 2C, Table IV).

Genes listed in Table I-IV were sorted according to their functions, and the genes whose altered expression could promote tumor progression or reduce tumor suppression, favoring tumor growth were selected for further analysis (Table V). Upregulation of the gene encoding amyloid βA4 precursor protein-binding family B member 2, which has been reported as an apoptosis inhibitor in aneuploid fibrosarcoma (17), was detected. The gene encoding Rho/Rac guanine nucleotide exchange factor 2, which is reported as an oncogenic protein in pancreatic cancer (18) and associated with the invasion and metastasis of breast cancer (19),

was also upregulated. Previously, interleukin 1α (IL1A) and interleukin 1β (IL1B) have been reported as being associated with the growth of lung cancer cells (20). Significant upregulation of the *IL1A* and *IL2B* genes was observed in the present study, which implied that the tumor microenvironment of resistant cells was altered. Leukemia inhibitory factor (encoded by the *LIF* gene) has been known to promote tumor progression and radioresistance in nasopharyngeal carcinoma (21). Among the upregulated genes, mouse double minute 2 homolog is a well-known oncogene that can promote tumor formation by targeting tumor suppressor proteins, including p53, for proteasomal degradation (22). The protein tyrosine phosphatase receptor type C gene, which is associated with the recurrence of colorectal cancer (23), was also upregulated. The stratifin gene, a gene which was upregulated in the present study, is associated with resistance to chemotherapy and radiotherapy in pancreatic cancer (24) and to tumor invasion in lung cancer (25). The vascular endothelial growth factor A gene, which is associated with angiogenesis in cancer (26) and the migration and invasion of lung cancer (27), was also upregulated.

Among the downregulated genes, the growth arrest-specific 1 gene, which is known to be a tumor suppressor gene (28), was detected. The poly (rC) binding protein 4 gene, which is induced by the p53 tumor suppressor and whose product suppresses cellular proliferation by inducing apoptosis and cell cycle arrest in G_2/M (29), was also downregulated. Decreased expression of the tumor necrosis factor gene encoding tumor necrosis factor, an inducer of apoptosis (30), was observed.

Table II. Altered expression of genes involved in apoptosis and the cell cycle.

Gene symbol	Description	Genbank	Fold change (vs. PC-9)	
			PC-9G	PC-9E
Upregulated				
APBB2	Amyloid β (A4) precursor protein-binding, family B, member 2	NM_004307	2.09	1.55
Downregulated				
BUB1B	BUB1 mitotic checkpoint serine/threonine kinase B	NM_001211	-1.65	-1.45
FOXC1	Forkhead box C1	NM_001453	-1.79	-2.06
ID3	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	NM_002167	-1.51	-1.75
PCBP4	Poly(rC) binding protein 4	NM_033010	-2.24	-1.54
SKP2	S-phase kinase-associated protein 2, E3 ubiquitin protein ligase	NM_032637	-2.25	-2.77

Table III. Altered expression of genes involved in the cell cycle and cellular proliferation.

Gene symbol	Description	Genbank	Fold change (vs. PC-9)	
			PC-9G	PC-9E
Upregulated				
CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	NM_004936	1.64	2.74
LIF	Leukemia inhibitory factor	NM_002309	1.59	2.24
MDM2	MDM2 proto-oncogene, E3 ubiquitin protein ligase	NM_002392	1.66	2.35
Downregulated				
PIN1	Peptidylprolyl cis/trans isomerase, NIMA-interacting 1	NM_006221	-1.87	-1.47
PRKCQ	Protein kinase C, theta	NM_006257	-1.78	-3.08

Discussion

Gefitinib and erlotinib, commonly considered as the standard treatment for patients with NSCLC who harbor *EGFR* activating mutations, are two oral drugs that bind the ATP-binding site in the tyrosine kinase domain of the EGFR protein. Somatic mutations occurring in the tyrosine kinase domain of the *EGFR* gene and responsible for ligand-independent activation of the EGFR pathway have been reported; exon 19 deletions and L858R substitution in exon 21 are the most common, accounting for 90% of all *EGFR* activating mutations in NSCLC (2,3). These mutations lead to increased growth signaling, thus conferring susceptibility to treatment with TKIs. Despite the initial clear responses to EGFR TKIs and substantial increase in progression free survival observed in various clinical trials, the majority of patients with NSCLC with an *EGFR* activating mutation develop acquired resistance subsequent to a median of ~12 months from treatment initiation (4). Although significant advances have been made, in ≤30% of cases the acquired resistance mechanisms remain unexplained. Therefore, there is an urgent need to identify the underlying mechanisms of resistance in order to develop effective targeted therapies for cancer progression subsequent to EGFR TKI treatment.

The present study established two NSCLC cell lines resistant to the commonly used EGFR TKIs, gefitinib or erlotinib. These cell lines harbor the *EGFR* T790M mutation, which is the most frequently reported mechanism of acquired resistance to EGFR TKI treatment. The present study aimed to analyze possible gene expression changes associated with EGFR TKI resistance using PC-9G and PC-9E cell lines. Among the genes whose expression was significantly altered, those whose expression was altered in PC-9G and PC-9E cells were focused on, in order to increase the reliability of the results.

It is known that when binding to one of its several ligands, EGFR forms homodimers or heterodimers with other members of the ERBB family receptor tyrosine kinases, and activates downstream pathways, including the phosphatidylinositol 3-kinase/protein kinase B, Raf/mitogen activated protein kinase/extracellular signal-regulated kinases, and janus kinase/signal transducers and activators of transcription signaling pathways, initiating a cascade of signaling events that trigger anti-apoptotic signaling, cellular proliferation,

Table IV. Altered expression of genes involved in cellular proliferation, apoptosis and also in the cell cycle.

Gene symbol	Description	Genbank	Fold change (vs. PC-9)	
			PC-9G	PC-9E
Upregulated				
IL1A	Interleukin 1, α	NM_000575	3.47	23.92
IL1B	Interleukin 1, β	NM_000576	2.68	17.35
PTPRC	Protein tyrosine phosphatase, receptor type, C	NM_001267798	3.61	2.25
SFN	Stratifin	NM_006142	3.10	3.39
VEGFA	Vascular endothelial growth factor A	NM_001025370	1.61	1.68
Downregulated				
ANG	Angiogenin, ribonuclease, RNase A family, 5	NM_001145	-1.50	-2.70
EGFR	Epidermal growth factor receptor	NM_201283	-1.79	-1.72
GAS1	Growth arrest-specific 1	NM_002048	-3.38	-2.77
JUN	Jun proto-oncogene	NM_002228	-1.81	-2.70
LTB	Lymphotoxin β (TNF superfamily, member 3)	NM_002341	-1.90	-2.70
PML	Promyelocytic leukemia	NM_033238	-1.62	-1.87
TGFB3	Transforming growth factor, β3	NM_003239	-2.18	-2.13
TNF	Tumor necrosis factor	NM_000594	-4.62	-2.35

Table V. Genes whose expression was altered towards enhanced tumor progression or reduced tumor suppression.

Gene symbol	Associated process	Fold change (vs. PC-9)	
		PC-9G	PC-9E
Tumor progression			
APBB2	Inhibition of tumor cell apoptosis	2.09	1.55
ARHGEF2	Oncogenic signaling, invasion and metastasis	1.52	2.29
IL1A	Growth of tumor cells	3.47	23.92
IL1B	Growth of tumor cells	2.68	17.35
LIF	Tumor progression and radioresistance	1.59	2.24
MDM2	Tumor formation	1.66	2.35
PTPRC	Cancer recurrence	3.61	2.25
SFN	Resistance to chemotherapy and radiotherapy, invasion	3.1	3.39
VEGFA	Angiogenesis, migration and invasion	1.61	1.68
Tumor suppression			
GAS1	Inhibition of cellular proliferation and induction of apoptosis	-3.38	-2.77
PCBP4	Suppression of cellular proliferation	-2.24	-1.54
TNF	Apoptosis of tumors cells	-4.62	-2.35

angiogenesis and tumor invasion and metastasis. Treatment with EGFR TKIs will induce blockage of these pathways. The present data indicated that EGFR TKI-resistant cells are likely to exhibit altered expression of genes that are associated with apoptosis, cellular proliferation and the cell cycle.

Genes, that are associated with at least two of the processes of cellular proliferation, apoptosis, and the cell cycle, and whose expression was significantly altered in EGFR TKI-resistant cells to favor tumor progression, are listed in Table V. As shown in Table V, there was increased expression of genes whose functions are known to promote tumor growth, and decreased expression of genes whose functions are known to suppress tumor growth, in PC-9G and PC-9E cells. It will be necessary to determine the action of these genes in the development of EGFR TKI resistance in NSCLC cells.
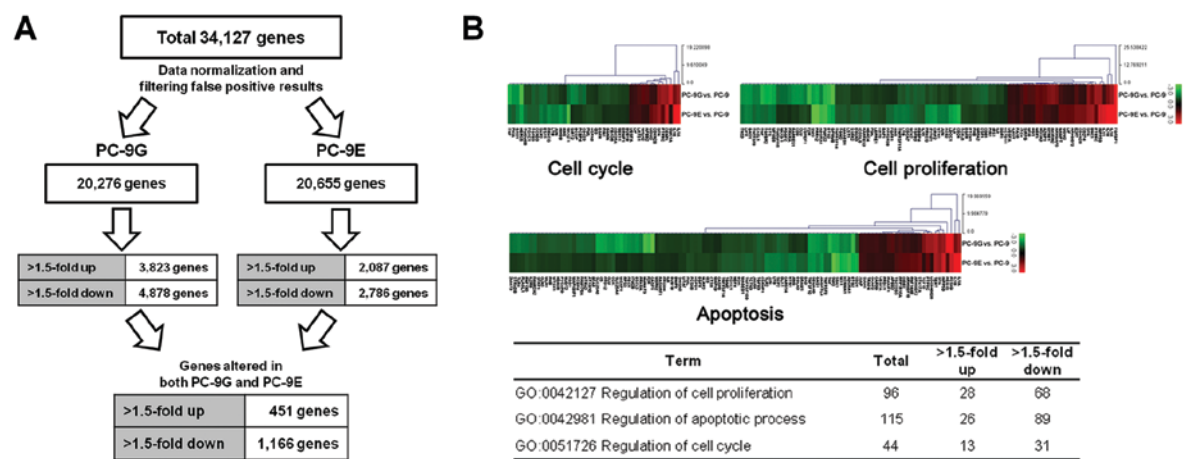
Figure 1. Clustering analysis of mRNA expression profiles of PC-9G and PC-9E cells. (A) Schematic workflow for analyzing gene expression altered in PC-9G and PC-9E cells by mRNA microarray analysis. (B) Gene ontology analysis (GOTERM_BP_ALL) was performed using the DAVID online tool. Functional annotation clustering identified a significantly up- or downregulated cluster of genes in PC-9G and PC-9E cells compared with PC-9 cells. Significantly altered expression of genes associated with the cell cycle, cellular proliferation and apoptosis is expressed by heat map. Transcriptional changes in PC-9G and PC-9E cells are expressed as the fold ratio compared with parental PC-9 cells.
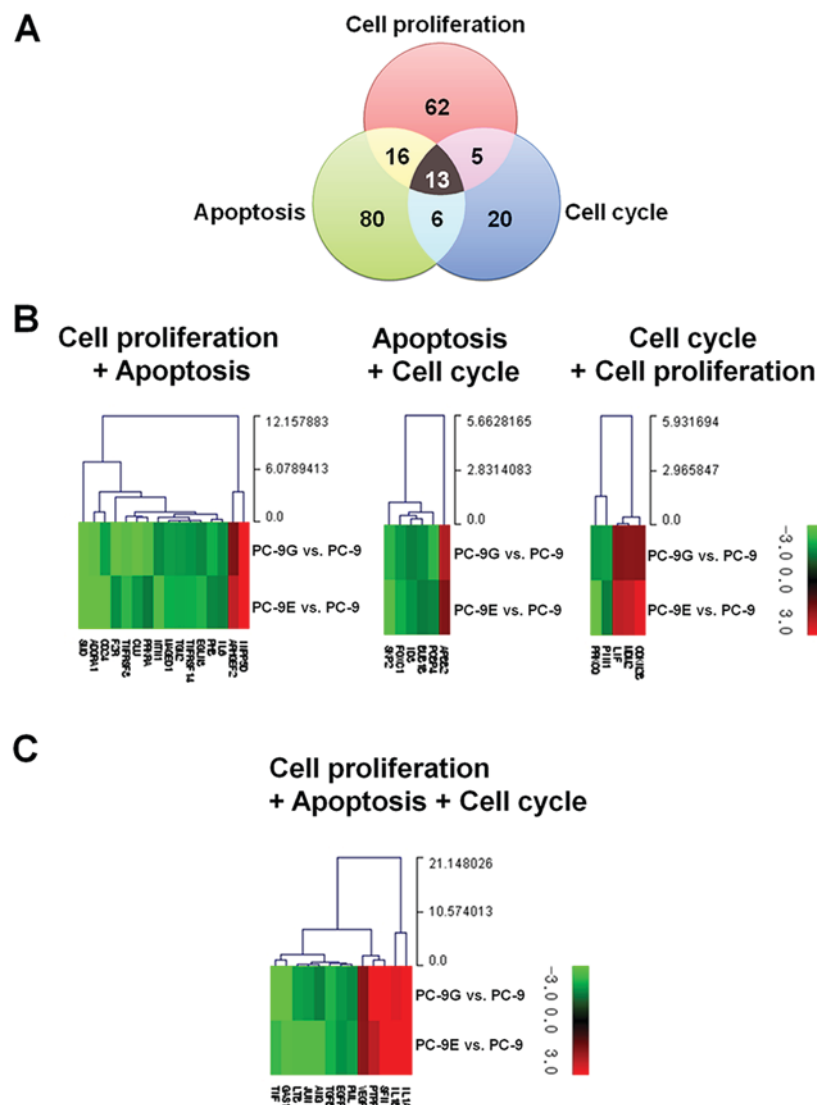

Figure 2. Functional pathways altered with the acquisition of resistance in PC-9G and PC-9E cells. (A) The number of genes common in any two or all three ontological clusters is given at the intersections of the circles. (B) Heat maps for the expression of genes associated with cellular proliferation and apoptosis, apoptosis and the cell cycle, and the cell cycle and cellular proliferation (right) are shown. (C) A heat map for the expression of genes involved in all of cellular proliferation, apoptosis and the cell cycle is presented. Transcriptional changes in resistant cells are expressed as the fold ratio compared with parental PC-9 cells.

Two previous studies were identified that had used mRNA microarray analysis to examine changes of gene expression in lung cancer cells with acquired resistance to either gefitinib or erlotinib (12,31). However, these studies only examined one cell line resistant to either gefitinib or erlotinib. The present study examined two cell lines with acquired resistance-one to gefitinib and one to erlotinib. Furthermore, genes whose expression was altered in the gefitinib- and the erlotinib-resistant cell line were investigated and the changes of gene expression in terms of major survival pathways were analyzed.

To the best of our knowledge, this is the first study to identify a common mRNA expression profile in cells with acquired resistance to either gefitinib or erlotinib, and can provide key insights into gene expression profiles involved in conferring resistance to EGFR TKI therapy in lung cancer cells. In conclusion, the present study observed distinctive gene expression patterns via mRNA microarray analysis of EGFR TKI-resistant lung cancer cells harboring the *EGFR* T790M mutation.

Acknowledgements

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