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Identification of key pathways and genes in lung carcinogenesis

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Abstract. The present study aimed to identify key pathways and genes in the pathogenesis of lung cancer. The GSE10072 dataset was downloaded from the Gene Expression Omnibus database. Protein-protein interaction data were collected from Human Protein Reference Database, and 201 pathways were downloaded from the Kyoto Encyclopedia of Genes and Genomes database. Signaling network impact analysis was performed to identify enriched pathways, followed by the construction of a pathway-pathway crosstalk network. Benzopyrene was used to treat normal human lung cells at concentrations of 0.01, 0.1, 1 and 10 μM, and cell viability was measured. Furthermore, growth arrest and DNA damage inducible β (GADD45B), p53, cyclin B, Akt and nuclear factor (NF)-κB protein levels were also measured via western blotting. Impact analysis identified 11 enriched lung cancer-associated KEGG pathways, including 'complement and coagulation cascades', 'ECM-receptor interaction', 'P53 signaling pathway', 'cell adhesion molecules' and 'focal adhesion'. In addition, cell cycle, 'drug metabolism-cytochrome P450', 'metabolic pathways', 'pathways in cancer', 'focal adhesion' and 'antigen processing and presentation' were central in the pathway-pathway cross-talk network. Furthermore, the upregulated gene GADD45B was associated with three of the pathways, including an activated pathway ('MAPK signaling pathway') and two repressed pathways ('cell cycle' and 'P53 pathway'). Western blotting demonstrated that the expression of NF-κB, Akt and GADD45B increased over time in lung cells treated with benzopyrene, whereas the expression levels of cyclin B and P53 decreased. In conclusion, GADD45B may contribute to lung carcinogenesis via affecting the MAPK, P53 signaling and cell cycle pathways.

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Introduction

Lung cancer is one of the most common types of cancer (1). Lung cancer has two main histological types: Non-small cell lung cancer (NSCLC; 80.4%) and SCLC (16.8%) (2); however, the underlying mechanisms for the development of lung cancer are not yet completely characterized.

Accumulated genetic abnormalities are associated with cancer. Somatic mutations in a number of genes, including epidermal growth factor receptor (*EGFR*), *P53*, *KRAS*, *BRAF*, Erb-B2 receptor tyrosine kinase 2 (*ERBB2*), *MET*, serine/threonine kinase 11, PIK3CA and Parkin RBR E3 ubiquitin protein ligase, have been identified in patients with lung cancer (3,4). Gene amplifications, including of *EGFR*, *ERBB2*, *MET*, *PIK3CA* and NK2 homeobox 1, have also been detected in lung cancer (5). A number of single nucleotide polymorphisms (SNPs) are associated with lung cancer susceptibility, including in interleukin-1, cytochrome P450, a 5'SNP in the ERCC excision repair 6, chromatin remodeling factor gene and SNPs in the nicotinic acetylcholine receptor gene cluster on chromosome 15q25.1 (6-9). Genetic abnormalities have been identified in numerous pathways, including the Notch (10), EGFR (11), PI3K (12), phosphatase and tensin homolog/phospho-Akt/P53 (13), mitogen-activated protein kinase (MAPK) (14) and cell cycle pathways.

In the past decade, there has been a pervasive application of high-throughput molecular technologies, including microarrays, in lung cancer research (15-17), which has greatly enriched the knowledge of the pathogenesis of the disease, and may potentially provide markers for the prognosis and targeted therapy of lung cancer. By enrolling 105 subjects in an Environment And Genetics in Lung cancer Etiology study (http://dceg.cancer.gov/eagle), Landi *et al* (18) produced a microarray dataset that included 107 expression values from tumor (n=58) and non-tumor tissues (n=49) from 74 subjects (non-smokers, n=20; former smokers, n=26; current smokers, n=28). Using this microarray analysis, 122 genes were identified that were differentially expressed between the tumor and non-tumor samples. In addition, several crucial smoking-associated genes and pathways were identified in the study, and a number of these, including Nima related kinase 2 and TTK protein kinase, were experimentally validated; however, the relationship between these genes and pathways were not considered in the original study.

In the present study, based on the microarray dataset produced by Landi *et al* (18), GSE10072, a novel pathway-pathway

crosstalk approach was employed to identify pathways and genes that may have critical roles in the pathogenesis of lung cancer, and a number of the identified genes were experimentally validated.

Materials and methods

Source of pathway and microarray data. Protein-protein interaction data were downloaded from the Human Protein Reference Database (http://www.hprd.org/), and 201 lung cancer pathways were downloaded from the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.kegg.jp/) database (19,20) using 'lung cancer' as the search term.

The raw data of the gene expression profile dataset GSE10072 in the '.CEL' format were downloaded from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/).

Identification of differentially expressed genes (DEGs). The raw downloaded data were preprocessed and normalized using the R/Bioconductor package Affy with the Robust Multichip Average method for single-channel Affymetrix chips (21). A one-way analysis of variance was applied to each probe set to identify those that significantly changed expression level over time, as previously described (22). P<0.05 was considered to indicate a statistically significant result; the raw P-value was adjusted with the Bonferroni method (23).

Impact analysis. The pathway impact analysis as described by Draghici *et al* (24) was adopted, which considers the statistical significance of the enrichment of KEGG pathways, while also considering other crucial factors, including the magnitude of expression change for each gene, the topology of the signaling pathway, and the interactions between signaling pathways.

Construction of pathway-pathway crosstalk network. A hyper geometric distribution framework was applied to evaluate the significance of all non-empty intersections between two pathways, as previously described (25): Fisher's exact test computed the probability, p^* using hyper geometric distribution with the parameters (S, N_G, N).

$$p^* = p(X = \alpha \mid S, N_G, N) = \frac{\binom{S}{\alpha}\binom{N-S}{N_G-\alpha}}{\binom{N}{N_G}}$$

Where α was the number of DEGs in the pathway intersection; S, the number of DEGs in the pathway union; N_G, the number of genes in the pathway intersection; and N, the number of genes in the pathway union.

The P-value to reject the null hypothesis with a probability of $<p^*$ was calculated using the sum of the probabilities with the same marginal totals, i.e.:

$$p = \sum \frac{\binom{S}{i}\binom{N-s}{N_G-i}}{\binom{N}{N_G}}$$

Table I. Significantly enriched Kyoto Encyclopedia of Genes and Genomes pathways in GSE10072.

Pathway	Impact factor	P-value
Complement and coagulation cascades	17.258	2.08×10^{-7}
ECM-receptor interaction	14.266	3.90×10^{-6}
P53 signaling pathway	9.017	3.41×10^{-4}
Cell adhesion molecules	70.734	4.42×10^{-4}
Focal adhesion	9.283	1.17×10^{-3}
Cell cycle	6.673	3.36×10^{-3}
Renin-angiotensin system	5.778	8.30×10^{-3}
PPAR signaling pathway	5.733	1.54×10^{-2}
TGF-beta signaling pathway	6.352	2.24×10^{-2}
Leukocyte transendothelial migration	114.039	2.34×10^{-2}
migration Tight junction	6.133	4.85×10^{-2}

This procedure gave a two-tailed probability for a Fisher's exact test. P<0.05 was considered to indicate a statistically significant result, indicative of the association of two pathways.

Analysis of cross-talk between pathways. The state of a pathway was initially determined. A pathway was considered to be activated when it met the following criteria: i) Number of DEGs in the pathway >10; ii) Q-value [(number of upregulated DEGs in the pathway-number of downregulated DEGs in the pathway)/total number of genes in the pathway] >0.5. A repressed pathway met the two criteria: i) Number of DEGs in this pathway >10; ii) Q-value <-0.5. Next, pairs of pathways sharing common DEGs were identified, and these DEGs were listed.

Cell culture and treatment. Normal lung K562 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin and streptomycin, in a humidified atmosphere with 5% CO_2 at 37˚C. To maintain drug resistance, adriamycin was supplemented at regular intervals for 2 weeks prior to any experiment. Benzopyrene was used to treat the cells at concentrations of 0.01, 0.1, 1 and 10 μM in the subsequent assays.

Measurement of cell viability. Cell proliferation was measured using an MTT assay. Cells (2×10^5 cells/ml) were seeded in 96-well plates with increasing concentrations of adriamycin, SNX-2112 and 17-AAG. After incubation at 37˚C for 24, 48 and 72 h, 5 mg/ml MTT solution was added for incubation for 4 h. Then, 100 μl/well DMSO was added to solubilize the formazan crystals. Cell viability was assessed by measuring absorbance at 570 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Western blot analysis. Cells were first lysed in lysis buffer (Sangon Biotech Co., Ltd., Shanghai, China) and then total

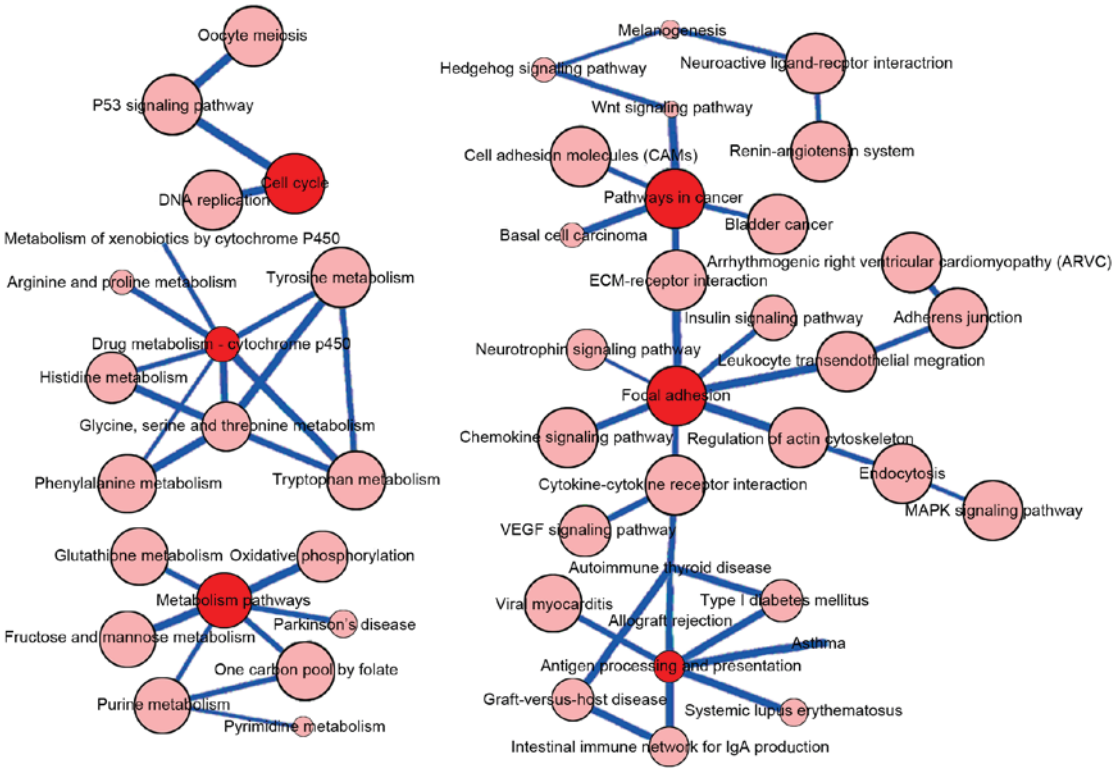

Figure 1. Network of pathway-pathway crosstalk for the GSE10072 dataset. The nodes represent each pathway, with the edges representing the crosstalk between the pathways. The thickness of the edges represents the strength of the pathway interactions. Red and pink represent primary and secondary nodes respectively.

protein was extracted. Followed by protein concentration was measured by the bicinchoninic acid method. Equal aliquots (20 μg protein per lane) of protein lysate were separated by 8-12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Subsequent to blocking with 5% skimmed milk at 37˚C for 1 h, Western blots were probed with primary antibodies against nuclear factor (NF)-κB (1:1,000; cat no. ab32360; Abcam, Cambridge, MA, USA), Akt (1:1,000; cat no. ab126811; Abcam), cyclin B (1:1,000; cat no. ab18221; Abcam), P53 (1:1,000; cat no. ab21985; Abcam), growth arrest and DNA damage inducible β (GADD45B) (1:1,000; cat no. ab128920; Abcam) and β-actin (1:1,000; cat no. ab6276; Abcam) overnight at 4˚C. Three consecutive washes were performed for 10 min in PBS-Tween, followed by incubation with the alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody (1:5,000; cat no. ab6722; Abcam) diluted in 5% skimmed milk at room temperature for 1 h. The immunoblots were visualized with enhanced chemiluminescence (GE Healthcare, Chicago, IL, USA) and autoradiography. The experiments were repeated three times, and the results were detected using the Image Lab software (version 4.1; Bio-Rad Laboratories, Inc.) on ChemiDoc MP imaging system (Bio-Rad Laboratories, Inc.).

Statistical analysis. All data were presented as the mean ± standard deviation. Statistical analysis was performed by one-way analysis of variance with a Bonferroni post hoc test in SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of DEGs. Using a threshold of P<0.05, the genes with significantly differential expression were analyzed. A total of 1,763 DEGs were identified, of which 662 were associated with KEGG pathways.

Result of impact analysis. A total of 11 pathways were identified in the pathway impact analysis. Of these, 'complement and coagulation cascades', 'ECM-receptor interaction', 'P53 signaling pathway', 'cell adhesion molecules (CAMs)', 'focal adhesion' and 'cell cycle' were the top five pathways by impact factor value (Table I).

Analysis of cross-talk between pathways. It was determined that the 'cell cycle', 'drug metabolism-cytochrome P450', 'metabolic pathways', 'pathways in cancer', 'focal adhesion' and 'antigen processing and presentation' KEGG pathways were central in the pathway-pathway crosstalk network (Fig. 1).

Using the criteria defined for activated and repressed pathways, 2 pathways ('cell cycle' and 'P53 signaling pathway') were repressed, and 10 pathways were activated (Table II). The common DEGs between the pathways are listed in Table III. *GADD45B* was associated with three enriched pathways, including the activated 'MAPK signaling pathway' and the repressed 'cell cycle' and 'P53 signaling pathway' terms (Fig. 2).

Measurement of cell viability and western blot analysis. Benzopyrene treatment reduced the viability of lung cells at

Table II. Status of the Kyoto Encyclopedia of Genes and Genomes pathways associated with the differentially expressed genes in lung cancer.

	DEGs			
Pathway	Upregulated	Downregulated	Both	Q-value
Cell cycle	2	11	13	-0.6923
P53 signaling pathway	2	8	10	-0.6000
Cytokine-cytokine receptor interaction	13	3	16	0.6250
Tight junction	9	2	11	0.6364
Neuroactive ligand-receptor interaction	15	3	18	0.6667
Chemokine signaling pathway	11	2	13	0.6923
Complement and coagulation cascades	14	2	16	0.7500
Axon guidance	9	1	10	0.8000
Vascular smooth muscle contraction	12	1	13	0.8462
MAPK signaling pathway	13	1	14	0.8571
Regulation of actin cytoskeleton	11	0	11	1.0000
Endocytosis	10	0	10	1.0000

Table III. Differentially expressed genes associated with multiple enriched Kyoto Encyclopedia of Genes and Genomes pathways.

Pathway 1	Pathway 2	Gene IDs
Cytokine-cytokine receptor interaction	Chemokine signaling pathway	6359, 1524, 2921, 2920, 6387
Neuroactive ligand-receptor interaction	Vascular smooth muscle contraction	1906, 10203, 1909, 185
MAPK signaling pathway	Regulation of actin cytoskeleton	2264, 6237, 2263
MAPK signaling pathway	Endocytosis	2264, 7048, 2263
Regulation of actin cytoskeleton	Endocytosis	2264, 2263, 8395
Tight junction	Regulation of actin cytoskeleton	10398, 6237, 4628
Cytokine-cytokine receptor interaction	Neuroactive ligand-receptor interaction	3953, 2690
Cytokine-cytokine receptor interaction	Chemokine signaling pathway	10563, 9547
Tight junction	Vascular smooth muscle contraction	10398, 4629
Tight junction	MAPK signaling pathway	6237, 10000
Vascular smooth muscle contraction	Regulation of actin cytoskeleton	10398, 4638
Cell cycle	P53 signaling pathway	4616
Cell cycle	MAPK signaling pathway	4616
Chemokine signaling pathway	Axon guidance	6387
Chemokine signaling pathway	Vascular smooth muscle contraction	115
Chemokine signaling pathway	MAPK signaling pathway	10000
Chemokine signaling pathway	Regulation of actin cytoskeleton	5295
Chemokine signaling pathway	Endocytosis	2869
Cytokine-cytokine receptor interaction	Axon guidance	6387
Cytokine-cytokine receptor interaction	MAPK signaling pathway	7048
Cytokine-cytokine receptor interaction	Endocytosis	7048
Neuroactive ligand-receptor interaction	Complement and coagulation cascades	728
Neuroactive ligand-receptor interaction	Endocytosis	154
P53 signaling pathway	MAPK signaling pathway	4616
Tight junction	Chemokine signaling pathway	10000
Vascular smooth muscle contraction	MAPK signaling pathway	5319
Vascular smooth muscle contraction	MAPK signaling pathway	5321

all concentrations from 72 h (Fig. 3). GADD45B, P53, cyclin , Akt and NF-κB protein levels were detected by Western blotting (Fig. 4). Western blotting demonstrated that the expression of NF-κB, Akt and GADD45B increased over time

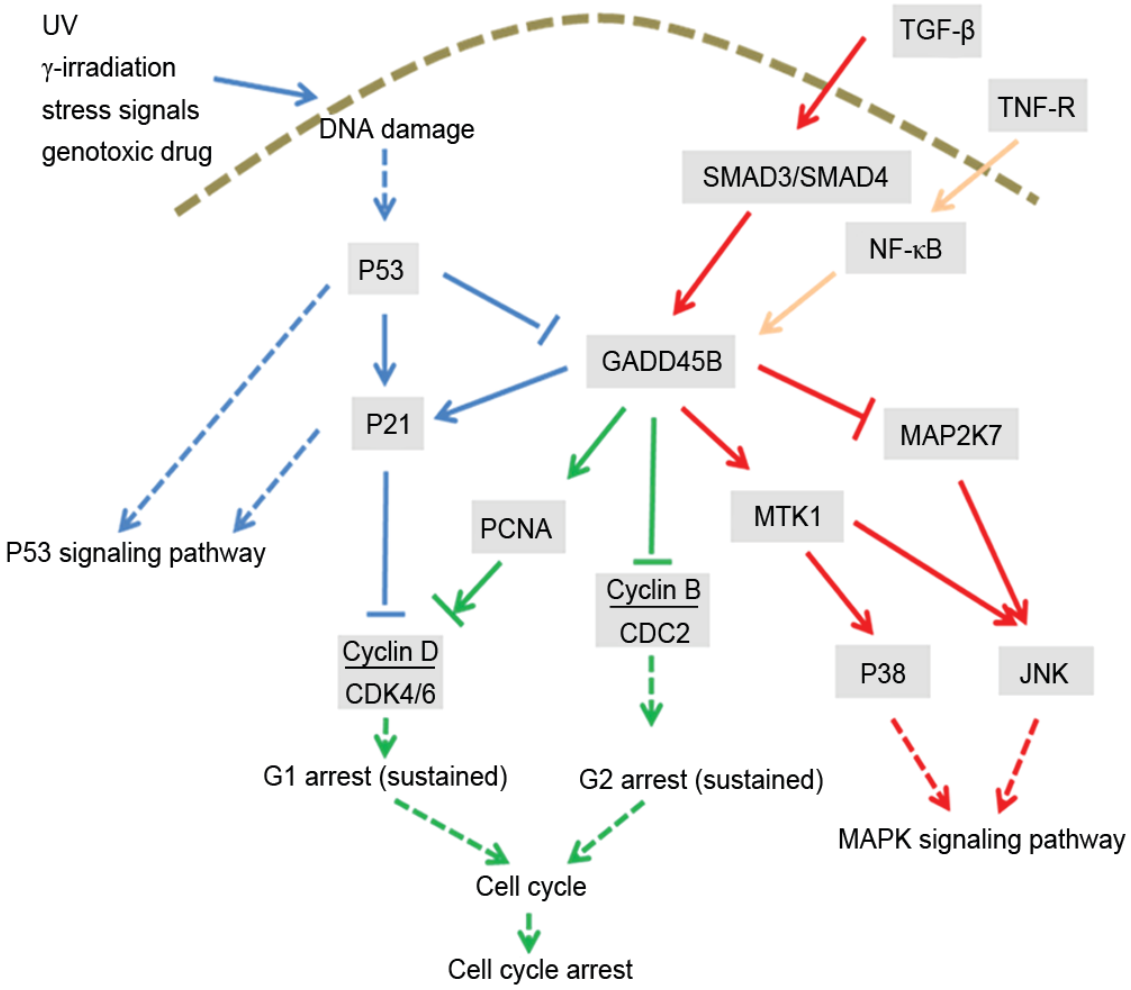

Figure 2. Schematic diagram of the pathway-pathway crosstalk via GADD45B. In this figure, the blue arrows represent the interaction with the P53 signaling pathway, the green arrows represent the interaction with the cell cycle pathway and the red arrows represent the interaction with the MAPK signaling pathway. Yellow arrows represent the interactions between GADD45B and genes that are not associated with 'P53 signaling pathway', 'MAPK signaling pathway' or 'cell cycle'. Solid lines represent direct interactions; dashed lines represent indirect effects. GADD45B, growth arrest and DNA damage inducible β; UV, ultraviolet radiation; TGF-β, transforming growth factor-β; TNF-R, tumor necrosis factor receptor; NF-κB, nuclear factor-κB; CDK, cyclin-dependent kinase; CDC2, cyclin-dependent kinase 1; PCNA, proliferating cell nuclear antigen; MAPK, mitogen-activated protein kinase; MTK1, MAPK kinase kinase 4; MAP2K7, MAPK kinase 7; JNK, c-Jun N-terminal kinase.

in lung cells treated with benzopyrene, whereas the expression of cyclin B and P53 was decreased (Fig. 5).

Discussion

In the present study, significantly enriched pathways were identified with impact analysis, and a pathway-pathway cross-talk network was constructed. GADD45B was identified as a connection between a number of the enriched pathways, and experimental validation of the expression of this gene and associated pathways was performed.

In comparison to classical pathway analysis, impact analysis also considers the over-representation of DEGs in a given pathway and the abnormal perturbation of that pathway, as measured by the expression changes across the pathway topology (26). Previously, the roles of the P53 signaling pathway, cell adhesion, focal adhesion and the cell cycle in the pathogenesis of lung cancer have been confirmed and studied extensively (27,28). In contrast, the complement and coagulation cascades in this disease have been less reported. Corrales *et al* (29), reported the activation of the complement system by detecting the anaphylatoxin C5a, a potent immune mediator generated subsequent to complement activation in lung cancer cell lines. Plasminogen activator inhibitor (PAI) variants *PAI-1* A15T and *PAI-2* S413C influence the prognosis of patients with lung cancer (30), and *PAI-1* has been demonstrated to inhibit the activation of the coagulation system (31). Levels of the erythrocyte complement receptor 1 were significantly lower in patients with small cell lung cancer (32). A previous study revealed that activated coagulation factor X inhibited the migration of lung cancer cells and may serve a key role in cell migration (33). Thus, it was indicated that the complement and coagulation cascades may also serve an important role in the pathogenesis of lung cancer.

The pathway-pathway cross-talk network indicated the central roles of 'cell cycle', 'drug metabolism-cytochrome P450', 'metabolic pathways', 'pathways in cancer', 'focal adhesion' and 'antigen processing and presentation'; metabolism-associated pathways may therefore be particularly important in the pathogenesis of lung cancer. Tumor cells sustain high

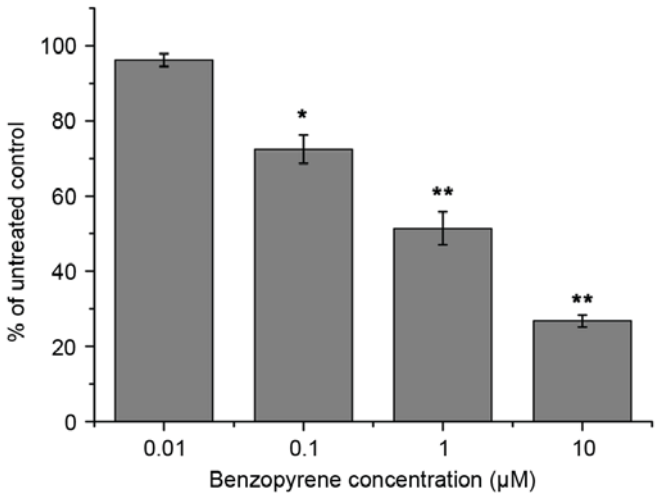

Figure 3. MTT assay of human bronchial epithelial cells cultured for 72 h with 0.01, 0.1, 1 or 10 μM benzopyrene. The data are presented as means ± standard deviation. *P<0.05 and **P<0.01 relative to the untreated control.

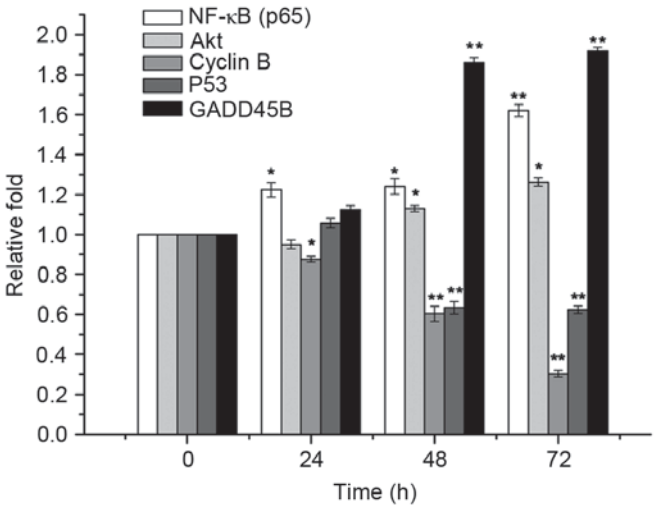

Figure 5. Quantification of the GADD45B, P53, cyclin B, Akt and NF-κB protein levels from the western blot analysis. *P<0.05 and **P<0.01 compared with 0 h. GADD45B, growth arrest and DNA damage inducible β; NF-κB, nuclear factor-κB.

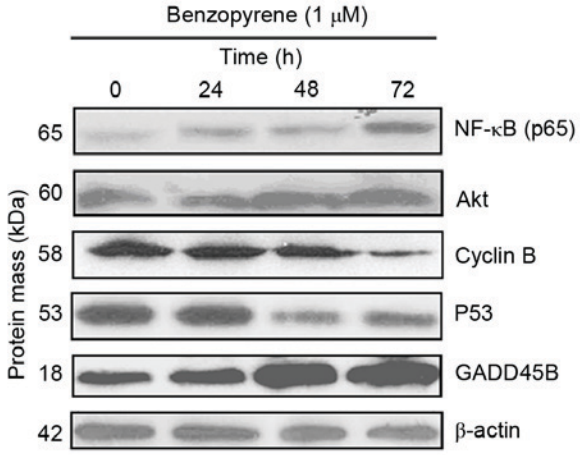

Figure 4. Identification of the GADD45B, P53, cyclin B, Akt and NF-κB protein expression levels using western blot analysis. GADD45B, growth arrest and DNA damage inducible β; NF-κB, nuclear factor-κB.

rates of glycolysis even in aerobic conditions to maintain their rapid growth; alterations in in primary metabolites (34) and glycolysis-associated enzymes, including hexokinase II and glyceraldehyde-3-phosphate dehydrogenase (35,36), have been identified in lung cancer. Additionally, the alteration of metabolic pathways may affect the efficacy of anti-tumor drugs, e.g. the glutathione metabolic pathway, which is involved in the detoxification or inactivation of platinum drugs (37). The cytochromes P450 are a group of enzymes that catalyze the oxidative biotransformation of the majority of drugs and other lipophilic xenobiotics (38).

In the present study, *GADD45B*, an upregulated gene, was determined to be a common link between three KEGG pathways, including the upregulated 'MAPK signaling pathway' and the repressed 'cell cycle' and 'P53 signaling pathway'. It was therefore considered to potentially have an important role in the pathogenesis of lung cancer. *GADD45B*, encoding MyD118, is part of a highly conserved Gadd45 gene family with *GADD45A*, encoding GADD45, and *GADD45G*, encoding CR6. Gadd45 proteins have been implicated for their involvement in tumorigenesis (39) and age-associated pathologies (40); however, MyD118, GADD45 and CR6 are considered to have similar but not identical functions via different apoptotic and growth suppressive pathways (41). The MAPK signaling pathway has been confirmed to have an important role in the pathogenesis of lung cancer (42). In the present study, this signaling pathway was activated in patients with lung cancer. Gupta *et al* (43) demonstrated that GADD45B promotes cell survival via activation of the GADD45a-P38-NF-κB pathway and inhibition of the MAPK kinase 4-c-Jun N-terminal kinases (JNK) pathway. P38 and JNK are members of the MAPK family (44). TGF-β can activate Smad by phosphorylation (45). One previous study demonstrates that following the induction by TGF-β, Smad transcription factors activate P38 via GADD45B (46). Another study demonstrates that SMAD3 and SMAD4 can activate GADD45B following the induction by TGF-β (47). GADD45B and GADD45G are cyclin-dependent kinase 2/cyclin B1 kinase inhibitors, and thus function in in G_2/M cell cycle arrest (48). This is in accord with the observation in the present study that the cell cycle pathway was repressed.

It was also identified in the present study that the P53 signaling pathway was repressed. Previously, Mi *et al* (49) observed the upregulation of GADD45B and the downregulation of P53 in human prostate cancer cells exposed to silvestrol. This signaling pathway has been reported to correlate with the radioresponse of NSCLC (13). GADD45A is a target gene of P53 (50). Lambert *et al* (51) reported the upregulation of GADD45B mRNA in Saos-2-His273 cells exposed to proline rich membrane anchor 1, a P53-reactivating agent.

Benzopyrene is a carcinogen particularly associated with lung cancer (52); therefore, the normal lung cells were treated with benzopyrene in the present study to investigate the process of lung carcinogenesis. The harmful effects of benzopyrene against cell viability were observed by an MTT assay at various concentrations. Western blotting demonstrated that the expression of NF-κB, Akt and GADD45B were increased over time in

lung cells treated with benzopyrene, whereas the expression of cyclin B and P53 were decreased. This corresponded with the data that the AKT-MAPK pathway was activated via NF-κB, while the P53 pathway and cell cycle pathway were repressed; thus, GADD45B may contribute to lung carcinogenesis via activating the MAPK signaling pathway and repressing the P53 signaling and cell cycle pathways.

Although a series of comprehensive bioinformatics analyses and validation experiments were performed, there were two major limitations in the present study: i) Only one lung cell line was used in the current study, which may not eliminate the heterogeneity of lung cancer; ii) knockdown of GADD45B was not performed, and the expressions of other proteins after GADD45B knockdown were not considered, which may weaken the regulatory associations between them.

In addition to 'ECM-receptor interaction', 'P53 signaling pathway', 'cell adhesion molecules (CAMs)', 'focal adhesion' and 'cell cycle', it was also identified that the 'complement and coagulation cascades' pathway may be associated with the pathogenesis of lung cancer; therefore, it was speculated that GADD45B may contribute to lung carcinogenesis via activating the MAPK signaling pathway, and repressing the P53 signaling and cell cycle pathways. Thus, the role of this gene in lung cancer should be studied further; GADD45B siRNA knockdown experiments will assist the further validation of this speculation.

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Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XJ and JL were involved in the conception and design of the research and drafting the manuscript. ZZ participated in the acquisition of data. RX performed the analysis and interpretation of data. YG was involved in the statistical analysis, XL participated in the design of the study and performed the statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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