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Screening of biomarkers for lung cancer with gene expression profiling data

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Abstract. – OBJECTIVES: Lung cancer is one of the most common malignant tumors, but the etiology is not yet clear. Our study aims to deepen the understandings about the mechanisms of lung cancer via screening relevant key genes and functional pathways.

MATERIALS AND METHODS: Microarray data set was collected and differentially expressed genes (DEGs) were selected out. KEGG pathway analysis and Gene Ontology (GO) enrichment analysis were performed for the DEGs. Interaction networks were constructed for the lung cancer-related DEGs with information from Human Protein Reference Database (HRPD) to screen out potential biomarkers.

RESULTS: Functional annotation revealed that cell cycle, DNA replication, immune system, and signal molecules and interactions were significantly over-represented in all the DEGs, suggesting their close involvement in the development of lung cancer. 40 genes with high degree, betweenness and clustering coefficient were identified from the interaction network. 26 out of them are known cancer genes according to the database F-census. Besides, 4 biomarkers were revealed through analyzing their interactions with oncogenes.

CONCLUSIONS: Our study not only advances the understandings about the molecular mechanisms of lung cancer, but also provides several potential biomarkers for clinical use.

Key Words:
Lung cancer, Functional enrichment analysis, Interaction network, Biomarker, CCNA2, CHEK1.

Introduction

Lung cancer is a common malignant tumor. Its incidence ranks first[1] among various cancers, and the number of new patients is 1.2 million per year in the world[2]. In recent years, there's a rapid increase in the incidence in China. A range of risk factors have been pointed out: (1) smoking, which contributes to almost 90% lung cancer death (90% for male and 85% for female) in developed countries[3]; (2) respiratory diseases, such as asthma, chronic obstructive pulmonary emphysema, pneumonia and tuberculosis; (3) air pollution; (4) psychological factors[4-6].

Like other cancers, alterations in genes have been found to be associated with lung cancer. Mutations in RAS, EGFR, Her2, c-MET, NKX2-1, LKB1, PIK3CA and BRAF increase the incidence[7-9]. Soriano et al[10] find that lung cancer cells utilise increased PI-9 expression to protect from granzyme B-mediated cytotoxicity as an immune evasion mechanism. Induced expression of B7-H4 also contributes to the immune escape of lung cancer cell[11]. Apart from explorations in mechanism, biomarkers discovery is another focus. They may serve as predictors and drug targets, and can be rapidly translated to clinical applications. A series of biomarkers have been reported, such as Nkx2-1[12], Interferon receptor 2[13], isocitrate dehydrogenase 1[14], protein phosphatase-1 D and growth arrest and DNA-damage-inducible-beta[15], as well as microRNAs like miR-125b[16], has-miR-339-5p[17]. Though the understandings have progressed much, more works are needed to fully disclose the underlying mechanisms and improve the outcomes of patients.

Microarray technology enables us to learn about the global changes in gene expression and, thus, uncover the regulatory mechanisms. In present study, we compared microarray data for cancer tissue samples with that for adjacent noncancerous tissue samples to screen out differentially expressed genes (DEGs). Key biological functions and pathways were also investigated to describe their roles in the whole process. In addition, several genes closely related with known cancer genes were also acquired, which might be potential therapeutic targets for future drug development.

Materials and Methods

Microarray Data

Microarray data set GSE19188[18] was downloaded from Gene Expression Omnibus (GEO) database, including 91 lung cancer samples and 65 adjacent non-carcinoma samples. Gene expression data was collected by Affymetrix Human Genome

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U133 Plus 2.0. Pretreatment, background correction and RMA (robust multiarray average) normalization were performed with package Affy of *R*[19, 20]. Probes linked with more than one Entrez Gene were deleted and those corresponding to one Entrez Gene were averaged. Finally, a total of 19804 genes were obtained.

Screening of Differentially Expressed Genes (DEGs)

DEGs between lung cancer tissue and surrounding tissue were selected out with Student's *t* test. BH (Benjamini and Hochberg) multiple testing correction was applied to control the false positive. FDR (False Discovery Rate) of < 0.05 and fold-change of > 2 were set as the cut-offs to determine DEGs.

Functional Enrichment Analysis

KEGG pathway analysis and Gene Ontology (GO) enrichment analysis was performed with DAVID[21] for the DEGs to identify altered biological functions in lung cancer. EASE method was adopted and EASE of < 0.1 was selected as the cut-off.

Establishment of Interaction Network

Proteins work together to exert certain functions, and alterations in the networks usually suggests the development of disease. Therefore, interaction network was established with information from Human Protein Reference Database (HPRD)[22] to observe the distribution of DEGs and thus disclose the underlying molecular mechanisms.

Interactions with Cancer Genes

Interactions between genes of interest and cancer genes were retrieved to better characterize their roles in the occurrence of disease. F-Census collects information about cancer-related genes from 6 literature search-based databases (CGC, OMIM, AGCOH, TGDBs, TSGDB and Cancer Genes) and candidate cancer genes predicted by high throughput sequencing, and it has gathered 2104 cancer genes[23]. Interactions were tested with Fisher model.

Results

Differentially Expressed Genes

Student's *t* test was chosen to identify DEGs between cancer tissue and adjacent tissue. A total of 1318 genes meeting the criteria of FDR < 0.05 and fold-change ≥ 2 were selected out as the DEGs.

Lung Cancer-Related Biological Process

KEGG pathway analysis was performed for the 1318 DEGs with DAVID online tools to identify disturbed biological functions. 9 terms meeting the cut-off (EASE score < 0.01) were obtained (Table I).

As shown in Table I, DEGs were enriched in following function groups: (1) cell cycle (cell cycle, p53 signaling pathway); (2) DNA replication; (3) immune function (complement and coagulation cascades, hematopoietic cell lineage); (4) signaling molecules and interactions (cytokine-cytokine receptor interaction, cell adhesion molecules (CAMs), ECM-receptor interaction).

GO enrichment analysis was also conducted for the DEGs. A total of 74 terms were significantly over-represented with FDR < 0.05 as cut-off. Significantly altered function terms were: cell cycle-related terms (cell cycle, M phase); immune-related terms (immune response, inflammatory response, humoral immune response, defense response, innate immune response); blood

Table I. Nine significantly enriched terms obtained from the DEGs

Term	Count	*p* value
hsa04610: Complement and coagulation cascades	26	2.18E-11
hsa04110: Cell cycle	23	2.61E-04
hsa04060: Cytokine-cytokine receptor interaction	38	3.08E-04
hsa04514: Cell adhesion molecules (CAMs)	22	0.001412964
hsa04640: Hematopoietic cell lineage	16	0.002678259
hsa04512: ECM-receptor interaction	15	0.005605304
hsa03030: DNA replication	9	0.005992305
hsa05310: Asthma	8	0.006200049
hsa04115: p53 signaling pathway	13	0.006308581

vessel development-related terms (blood vessel development, blood vessel morphogenesis).

These terms were closely associated with the pathogenesis of lung cancer, thus the 528 DEGs enriched in these terms were gathered for further analysis.

Protein-Protein Interaction Network

Interaction network was established for the proteins related with lung cancer with information from HPRD to further uncover the mechanisms. The construction process was as follow: (1) interaction information was extracted from the database, including 9518 genes and 37041 interactions after removing self-interactions; (2) the common genes between the 9518 genes and those in Human Genome U95 Version 2 Array were picked out; (3) the 582 DEGs were entered to establish the network. 456 out of them had annotations in the network with a total of 3949 interactions. Then the interaction network was depicted with cytoscape, see Figure 1.

As shown in Figure 1, these genes of interest were closely connected and most of them were located in the center of the network. To better describe their characteristics in the network, degree, betweenness and clustering coefficient were calculated. 55 genes with degree no less than 20 were observed. This was in accordance with previous study[24]. The 55 genes also showed big betweenness. A total of 40 genes with betweenness more than 0 were then selected out. These genes mediated several pathways and thus played important roles in maintaining cellular functions. In addition, high clustering coefficient means these genes tend to work together to exhibit certain functions. Overall, these genes presented similar characteristics as oncogenes and were listed in Table II.

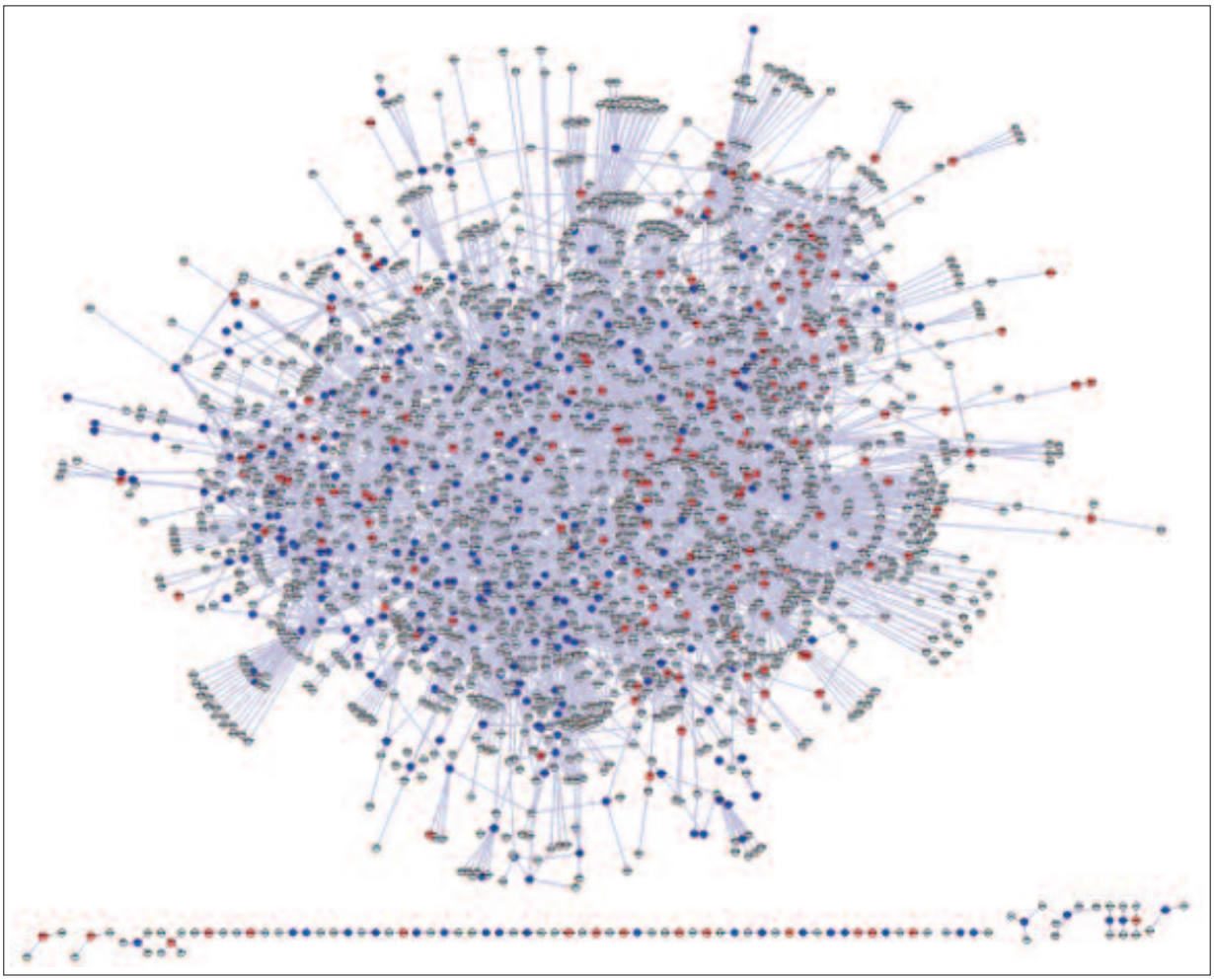

Figure 1. The interaction network for the 456 DEGs. The red dots represent up-regulated gene and blue dots down-regulated gene. The edges represent interaction.

Table II. The characteristics for the 40 genes in the network.

Gene	Degree	Betweenness	Clustering coefficient
CDK1	119	510563.8	0.007691
VIM	111	352674.8	0.002129
ZBTB16	82	239569.8	0.001204
CAV1	73	258030.1	0.004566
NFKBIA	60	168305.9	0.000565
HCK	55	116203.1	0.010774
KIT	54	105636.3	0.006988
FOS	52	111799.9	0.001508
KRT15	48	80450.89	0.001773
PLK1	47	98110.46	0.008326
SMAD7	43	99828.57	0.003322
LRP2	43	92223.55	0.001107
KDR	42	109358.1	0.012776
CDKN2A	41	75437.52	0.020732
FHL2	38	112945.2	0.002845
MCM7	36	61345.78	0.088889
MCM2	35	40726.34	0.090756
TGFBR2	34	131635.5	0.02852
MMP9	30	52679.49	0.016092
CCNB1	29	30646.81	0.05665
COL1A1	29	86451.59	0.027094
CHEK2	28	42587.78	0.007937
PFN2	28	37141.86	0.002646
MCM10	27	43903.51	0.094017
CCNA2	27	36560.06	0.037037
IGFBP3	27	97725.42	0.017094
FGF2	27	76363.35	0.002849
C3	26	55059.62	0.018462
TP53	25	110871.7	0.033333
CHEK1	25	37940.65	0.006667
AURKA	23	54243.37	0.003953
DSP	22	39806.94	0.056277
PECAM1	22	44440.61	0.021645
GHR	22	18246.44	0.004329
CDC6	21	15960.59	0.142857
CDC20	21	36604.76	0.038095
CXCR2	21	38180.95	0.019048
CDH5	20	68488.56	0.036842
TOP2A	20	33413.75	0.026316
RACGAP1	20	34038.84	0.010526

Compared with the cancer gene database F-Census[23], we found that 26 out of the 40 genes were included in the database. Considering the left 14 genes might take important parts in the development of lung cancer, their interactions with cancer genes were investigated. Fisher model was adopted to examine their relationship with cancer genes and p value < 0.05 was set as the cut-off. Finally, 4 genes were found to be significant (Table III).

Table III. The 4 genes that closely associated with cancer genes.

Gene.Symbol	ENTREZ_GENE_ID	No. of interacting cancer genes	*p* value
CCNA2	890	21	7.39E-12
CHEK1	1111	16	2.10E-07
CDH5	1003	9	0.003243
TOP2A	7153	8	0.012554

The interactions between the 4 genes and cancer genes were depicted with cytoscape (Figure 2). There were 54 interactions in total. Interestingly, 3 of them formed a module with other genes and thus were worthy of further research.

Discussion

In present study, microarray data was analyzed to identify key genes and biological functions that played important roles in the pathogenesis of lung cancer. A total of 1318 DEGs were obtained. GO enrichment analysis revealed that DEGs were significantly enriched in terms like cell cycle, DNA replication, immune function, and signaling molecules and interactions. Then interaction network was established and 40 DEGs with high degree, betweenness and clustering coefficient were selected out. According to the cancer gene database F-Census, 26 of them were reported to be cancer genes. Besides, 4 genes closely interacted with cancer genes: cyclin A2 (CCNA2), checkpoint kinase 1 (CHEK1), DNA topoisomerase II alpha (TOP2A) and cadherin 5 (CDH5). They might take important parts in the development of lung cancer.

CCNA2 belongs to cyclin family. It activates CDC2 or CDK2 kinases, and thus promotes both cell cycle G1/S and G2/M transitions. Besides,

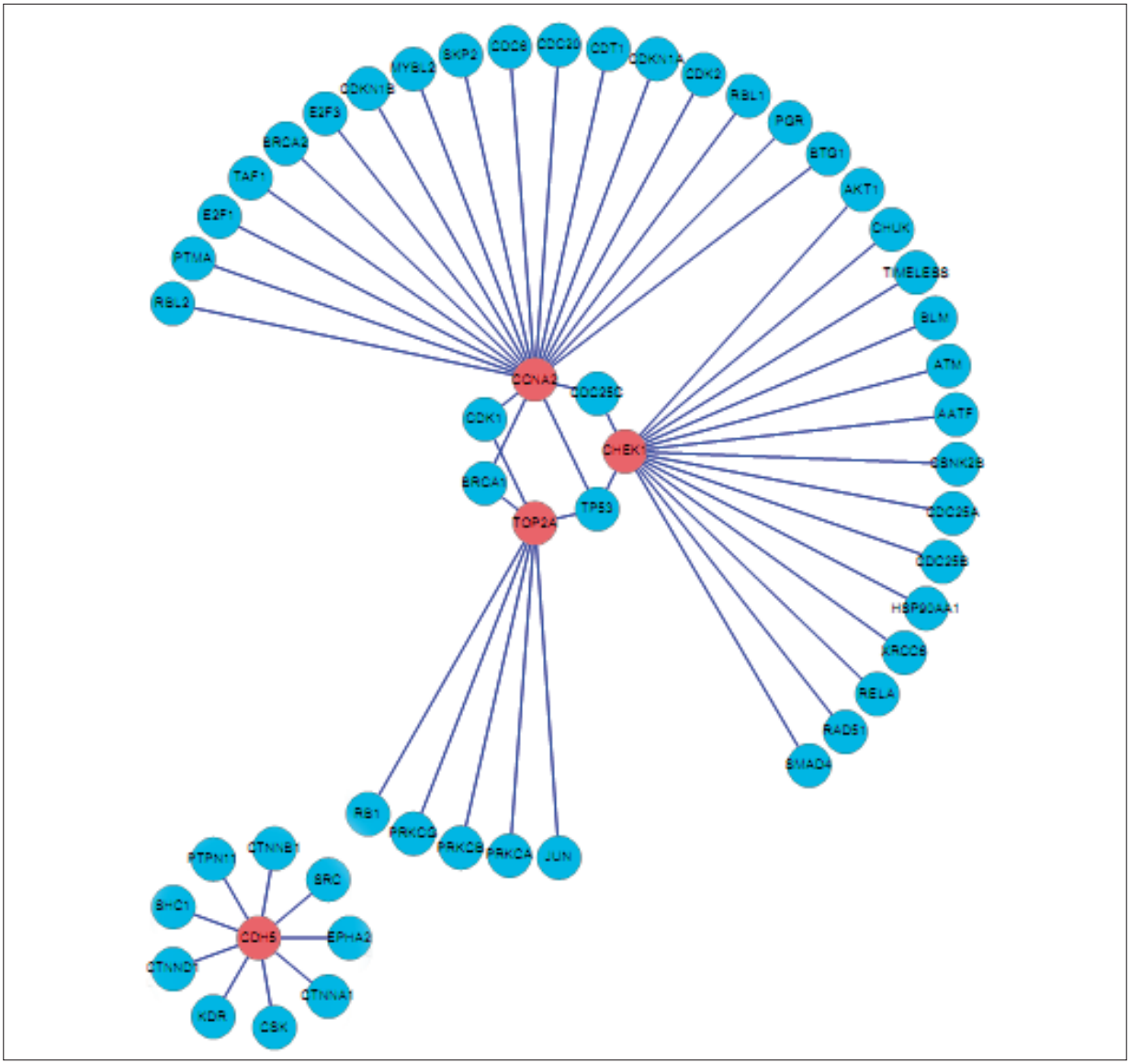

Figure 2. The interaction network for the 4 genes and cancer genes. The red dots represent the 4 genes and blue dots cancer genes.

it's also involved in cytoskeletal rearrangements and cell migration. Arsic et al[25] report that it negatively controls cell motility by promoting RhoA activation. It can serve as an predictor for recurrence risk in early breast cancer[26], but the prognostic significance of CCNA2 overexpression in non-small cell lung canceris controversial. Nevertheless, the study by Ko et al[27] demonstrates the synergistic effect of bcl-2 and cyclin A2 on adverse recurrence-free survival in stage I non-small cell lung cancer.

CHEK1 belongs to the Ser/Thr protein kinase family. It is required for checkpoint mediated cell cycle arrest in response to DNA damage or the presence of unreplicated DNA[28]. It is a promising drug target for treating cancers [29-32]. The study by Herman-Antosiewicz and Singh[33] confirms that diallyl trisulfide, an inhibitor for CHEK1, can inhibit proliferation of PC-3 and DU145 human prostate cancer cells. Yang et al[34] find that inhibition of CHEK1 sensitizes lung cancer brain metastases to radiotherapy. Of course, many small molecule drugs are being developed, such as LY2603618[35].

TOP2A is a DNA topoisomerase, an enzyme that controls and alters the topologic states of DNA during transcription. It functions as the target for several anticancer agents[36,37]. Lin et al[38] report that a cysteine-reactive alkyl hydroquinone modifies topoisomerase II±, enhances DNA breakage, and induces apoptosis in cancer cells. The study by Rijavec et al[39] shows that altered levels of topoisomerase IIα and BCRP in small cell lung cancer are important factors that contribute to resistance to chemotherapeutics that interfere with the enzyme and/or DNA and are highly associated with overall survival. In addition, a variety of mutations in this gene have been associated with the development of drug resistance. Kubo et al[40] report the resistance of etoposide in patients with point mutations in TOP2A.

CDH5 is a member of cadherin superfamily and involved in cell-cell adhesion. Its down-regulation has been reported in various human cancers[41-43], which subsequently promotes metastasis and invasion. Yuan et al[44] report decreased levels of E-cadherin mRNA and E-cadherin protein in the lung cancer tissues. The study by Shiwu et al[45] indicates that decreased expression of CD82/KAI1 and E-cadherin was closely related to cellular differentiation, pTNM stages, invasion and metastasis of non-small cell lung cancer. Its prognostic value has been investigated in several cancers[46,47]. Latest study by van Horssen et al[48] find that E-cadherin promoter methylation and mutation are inversely related to motility capacity of breast cancer cells.

In addition to the 26 oncogenes, these 4 genes are implicated in cancer development and thus can be good biomarkers for lung cancer. Additionally, CCNA2, CHEK1 and TOP2A are associated with cell cycle and form a module with other genes in the network. Their simultaneous alterations may contribute to the lung cancer to a considerable extent and thus worthy further researches.

Complement and coagulation cascades and p53 signaling pathway were among the enriched function groups. Lin et al[49] report that complement and coagulation cascades is one significant pathway of DEGs screened based on profile GSE10072 and GSE2514, but few studies have demonstrated a potential role of individual genes within this pathway in cancer development. As one of the most important cancer genes, p53 is involved in more 50% human malignant tumor. P53 signaling is found to be associated with lung adenocarcinoma using bioinformatics analysis[15]. Immune-related Go terms, such as immune response were enriched in this study, which was consistent with the study that immune response regulation is one enriched function of differentially methylated genes in lung cancer[50]. By functional enrichment analysis, we can detect lung cancer related biological process and can further clarify the etiology of it.

Conclusions

We adopted a series of bioinformatic tools to analyze microarray data and identify quite a few DEGs. Some of the DEGs are known oncogenes while some others are closely associated with cancer genes, especially the 4 genes. It proved the validity of our analysis strategy that combines functional enrichment analysis and interaction network analysis. More important, these findings are beneficial in advancing the understandings about pathogenesis of lung cancer and also provide potential biomarkers for diagnosis and/or treatment.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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