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LUNG CANCER

Identification of Commonly Dysregulated Genes in Non-small-cell Lung Cancer by Integrated Analysis of Microarray Data and qRT-PCR Validation

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Abstract
Background Non-small-cell lung cancer (NSCLC), the most common lung cancer, leads to the largest number of cancer-related deaths worldwide. There are many studies to identify the differentially expressed genes (DEGs) between NSCLC and normal control (NC) tissues by means of microarray technology. Because of the inconsistency of the microarray data sets, we performed an integrated analysis to identify DEGs and analyzed their biological function.
Methods and Results We combined 15 microarray data sets and identified 1063 DEGs between NSCLC and NC tissues; in addition, we found that the DEGs were enriched in regulation of cell proliferation process and focal adhesion signaling pathway. The protein–protein interaction network analysis for the top 20 significantly DEGs revealed that CAV1, COL1A1, and ADRB2 were the significant hub proteins. Finally, we employed qRT-PCR to validate the meta-analysis approach by determining the expression of the top 10 most significantly DEGs and found that the expression of these genes were significantly different between tumor and NC tissues, in accordance with the results of meta-analysis.
Conclusion qRT-PCR results indicated that the meta-analysis approach in our study was acceptable. Our data suggested that some of the DEGs, including MMP12, COL11A1, THBS2, FAP, and CAV1, may participate in the pathology of NSCLC and could be applied as potential markers or therapeutic targets for NSCLC.

Keywords Non-small-cell lung cancer · Meta-analysis · Microarray · Differentially expressed genes

Introduction

Lung cancer leads to the largest number of cancer-related deaths worldwide, more than 85 % of which are lung adenocarcinoma, also known as non-small-cell lung cancer (NSCLC) [1, 2]. The predicted 5-year survival rate of NSCLC patients is 15.9 %, and little improvement has been reached during the past few decades [3]. Due to the poor clinical outcome, substantial researches focus on uncovering the molecular mechanism of NSCLC, providing insights into potential therapeutic targets.

Microarray analysis is widely used in the field of cancer genetics research, which can measure gene expression on a genome-wide scale simultaneously [4]. The technology of the microarray helps to better understand the mechanisms of various diseases [5]. Previous studies have used this technique to find the differentially expressed genes (DEGs) between NSCLC and normal tissues. However, the results of these studies are inconsistent, probably due to sample sources, microarray platforms, and analysis techniques. In order to avoid these problems, a meta-analysis method is developed to detect DEGs by integrating multiple microarray studies [6]. This method has been performed in various types of tumors including hepatocellular carcinoma [7], nasopharyngeal carcinoma [8], colorectal cancer [9], and osteosarcoma [10] to detect key genes, i.e., oncogenes or tumor suppressor genes involved in the development of cancers.

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In this study, we employed meta-analysis method to identify DEGs between NSCLC and normal control (NC) tissues, and then we performed functional annotation of these genes to discover the biological processes and signaling pathways associated with NSCLC. Finally, we utilized qRT-PCR to validate the meta-analysis approach.

Materials and Methods

Strategy for Identification of NSCLC Gene Expression Datasets

We searched PubMed database and gene expression omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) database to identify NSCLC expression profiling studies by microarray. The following keywords and their combinations were used: lung adenocarcinoma and homo sapiens. The original studies that compared gene expression profiling between NSCLC and NC biopsy tissues or cultured cells were included in this study. Non-human studies, reviews, and meta-analysis articles were excluded.

After the background correction and normalization of raw data, we used significance analysis of microarray (SAM) to normalize the data and identified the DEGs by *t* test. The false discovery rate (FDR) <0.01 was selected as the criterion for significant differences.

Functional Classification of DEGs

We performed gene ontology (GO) enrichment analysis of the DEGs to investigate their biological functions in NSCLC using the online software GENECODIS (http://genecodis.cnb.csic.es) [11]. We also performed the pathway enrichment analysis by utilizing the Kyoto encyclopedia of genes and genomes (KEGG) database.

PPI Network Construction

The protein–protein interactions (PPIs) play central role in the regulation of biological processes and reveal the function of proteins at molecular level. The construction of PPI Network in a genome-wide scale is important for the interpretation of its functions. Biological general repository for interaction datasets (BioGRID) (http://thebiogrid.org/) was used to construct PPI network, and the top 10 up- and down-regulated DEGs were visualized in the network in Cytoscape [12].

The clinical specimens were provided by First Affiliated The Collection of Clinical Specimens

Hospital of PLA General Hospital, with the permission of the patients. Before the study, the protocols and documents were approved by the Medical Ethics Committee of the hospital. The written informed consent forms were obtained from the patients or legal guardians of the patients. The utilization of the samples was in strict accordance with the National Regulation of Clinical Sampling in China. The tumor tissues were immediately frozen and were stored in liquid nitrogen until RNA extraction.

RNA Preparation and qRT-PCR

Total RNA was extracted from each sample using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Two micrograms total RNA was reverse transcribed into single-stranded cDNA using superscript II reverse transcriptase (Invitrogen/Life Technologies, Carlsbad, CA). We utilized PrimerPlex 2.61 (PREMIER Biosoft, Palo Alto, CA) to design primers (see Supplementary Table 1 for primers used). cDNA was amplified using Power SYBR Green PCR Master Mix (Applied Biosystems/Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Quantitative PCR was performed with ABI 7500 real-time PCR system (Applied Biosystems, Carlsbad CA). The results were analyzed using Ct method using data assist software version 3.0 (Applied Biosystems/Life Technologies). The data were normalized to ACTIN gene expression.

Results

Differential Gene Expression Analysis by Meta-analysis

We identified 15 expression profiling studies eligible for the meta-analysis, including a total of 637 cases of NSCLC and 298 cases of NC. The details of the selected studies are summarized in Table 1. We found 1063 DEGs with FDR <0.01, among which 464 genes were up-regulated and 599 genes were down-regulated in NSCLC tissues. The top 20 significantly DEGs are listed in Table 2. The full list of the DEGs can be found in Supplementary Table 2.

Functional Annotation

We conducted a GO categories enrichment analysis to investigate the biological roles of identified DEGs. We separately examined the three groups of GO categories, including biological process, cellular component, and molecular function, by web-based software GENECODIS. Genes of $P < 0.01$ were selected and were tested against the background set of all genes with GO annotations. We found that the enriched GO terms for biological process was regulation of cell proliferation (GO: 0042127), and while for cellular component was plasma membrane part

Table 1 Characteristics of the individual studies

GEO ID	Platform	No. of genes	Samples (cancer: normal)
GSE1037	GPL962 CHUGAI 41K	8749	32:19
GSE1987	GPL91 [HG_U95A] Affymetrix Human Genome U95A Array	8503	28:9
GSE2088	GPL962 CHUGAI 41K	8729	57:30
GSE6044	GPL201 [HG-Focus] Affymetrix Human HG-Focus Target Array	8267	20:5
GSE7670	GPL96 [HG-U133A] Affymetrix Human Genome U133A Array	12,266	34:30
GSE8569	GPL5645 CNIO Human Oncochip 2.0	4238	69:6
GSE11969	GPL7015 Agilent Homo Sapiens 21.6K custom array	12,440	149:5
GSE18842	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	17,213	46:45
GSE19804	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	17,213	60:60
GSE21933	GPL6254 Phalanx Human OneArray	13,522	21:21
GSE23066	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	17,213	5:5
GSE27262	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array	17,213	25:25
GSE29249	GPL10558 Illumina HumanHT-12 V4.0 expression beadchip	18,565	6:6
GSE30118	GPL9365 Ocimum Biosolutions Human 40K OciChip	12,115	5:2
GSE43458	GPL6244 [HuGene-1_0-st] Affymetrix Human Gene 1.0 ST Array [transcript (gene) version]	18,564	80:30

Table 2 The top ten most significantly up- or down-regulated DEGs

Up-regulated genes			Down-regulated genes		
Gene ID	Gene symbol	*P* value	Gene ID	Gene symbol	*P* value
4321	MMP12	3.26E−67	2167	FABP4	1.19E−88
1301	COL11A1	3.46E−61	1003	CDH5	7.98E−82
7058	THBS2	1.39E−57	3777	KCNK3	7.56E−81
1311	COMP	8.93E−54	857	CAV1	8.70E−76
1277	COL1A1	2.87E−51	762	CA4	9.30E−76
8038	ADAM12	3.22E−47	10,266	RAMP2	1.57E−72
2191	FAP	1.16E−46	6943	TCF21	3.89E−71
1281	COL3A1	5.55E−46	154	ADRB2	1.13E−69
6518	SLC2A5	4.24E−44	10,268	RAMP3	2.95E−69
5099	PCDH7	7.24E−44	11,170	FAM107A	3.26E−67

(GO: 0044459), for molecular function was growth factor binding (GO: 0019838). The full list of GO terms is given in Table 3.

We also performed the KEGG pathway enrichment analysis to further explore the biological significance of the DEGs. Hypergeometric test with P value <0.05 was used as the criterion for pathway detection. From the KEGG pathway analysis, we found that 37 genes were enriched in focal adhesion signaling pathway, indicating that they may relate with NSCLC metastasis (Fig. 1).

PPI Network Construction

Based on PPI data in BioGRID, PPI networks of the top 20 significantly DEGs were constructed by Cytoscape software. The PPI network consisted of nodes and edges, which represents proteins and interactions. There were 410 edges and 404 nodes in the PPI network, among which ADRB2, CAV1, and COL1A1 were connected with more proteins (Fig. 2).

qRT-PCR Validation

Five pairs of NSCLC and NC tissues were used to validate the results of meta-analysis. We selected the top ten up- or down-regulated genes for validation. MMP12, COL11A1, THBS2, ADAM12, and FAP were selected as the up-regulated genes in NSCLC, while FABP4, CDH5, CAV1, TCF21, and ADRB2 were selected as the down-regulated genes in NSCLC.

The qRT-PCR results showed that the expression patterns of selected genes in NSCLC and NC tissues were similar to those in the meta-analysis. The expression

Table 3 The enriched GO categories of DEGs

GO ID	GO term	No. of genes	FDR
Biological process			
GO:0042127	Regulation of cell proliferation	134	1.08E−20
GO:0008284	Positive regulation of cell proliferation	73	1.45E−10
GO:0010033	Response to organic substance	100	1.23E−08
GO:0009611	Response to wounding	78	2.21E−07
GO:0008285	Negative regulation of cell proliferation	59	1.05E−06
GO:0006928	Cell motion	69	5.32E−06
GO:0008283	Cell proliferation	65	5.81E−06
GO:0009719	Response to endogenous stimulus	60	3.22E−05
GO:0048545	Response to steroid hormone stimulus	37	3.72E−05
GO:0009725	Response to hormone stimulus	56	3.72E−05
GO:0032101	Regulation of response to external stimulus	33	3.96E−05
GO:0006357	Regulation of transcription from RNA polymerase II promoter	90	5.16E−05
GO:0016477	Cell migration	46	5.88E−05
GO:0043067	Regulation of programmed cell death	96	1.56E−04
GO:0010941	Regulation of cell death	96	1.86E−04
GO:0042981	Regulation of apoptosis	95	1.89E−04
GO:0051674	Localization of cell	48	2.10E−04
GO:0048870	Cell motility	48	2.10E−04
GO:0001501	Skeletal system development	49	2.64E−04
GO:0010604	Positive regulation of macromolecule metabolic process	99	2.91E−04
GO:0033043	Regulation of organelle organization	38	3.09E−04
GO:0050727	Regulation of inflammatory response	20	9.13E−04
GO:0007155	Cell adhesion	83	0.001206291
GO:0022610	Biological adhesion	83	0.00126829
GO:0035295	Tube development	37	0.001273906
GO:0034097	Response to cytokine stimulus	20	0.001737386
GO:0045893	Positive regulation of transcription, DNA dependent	62	0.002032236
GO:0007242	Intracellular signaling cascade	129	0.00225627
GO:0051254	Positive regulation of RNA metabolic process	62	0.002716569
GO:0043627	Response to estrogen stimulus	23	0.003083008
GO:0051094	Positive regulation of developmental process	42	0.003628592
GO:0045944	Positive regulation of transcription from RNA polymerase II promoter	51	0.004275458
GO:0051173	Positive regulation of nitrogen compound metabolic process	76	0.004478782
GO:0007610	Behavior	60	0.005196598
GO:0042060	Wound healing	32	0.008897638
GO:0045935	Positive regulation of nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process	73	0.010140217
GO:0001944	Vasculature development	38	0.011500818
GO:0009891	Positive regulation of biosynthetic process	79	0.011875543
GO:0007584	Response to nutrient	26	0.013246813
GO:0050878	Regulation of body fluid levels	26	0.015085619
GO:0001568	Blood vessel development	37	0.016460382
GO:0007599	Hemostasis	22	0.018233448
GO:0048585	Negative regulation of response to stimulus	21	0.019014018
GO:0032583	Regulation of gene-specific transcription	25	0.019058568
GO:0048729	Tissue morphogenesis	30	0.020670543
GO:0010647	Positive regulation of cell communication	45	0.020959123
GO:0010628	Positive regulation of gene expression	68	0.022124705

Table 3 continued

GO ID	GO term	No. of genes	FDR
GO:0031960	Response to corticosteroid stimulus	19	0.022880876
GO:0045597	Positive regulation of cell differentiation	35	0.023186475
GO:0031328	Positive regulation of cellular biosynthetic process	77	0.023298999
GO:0048568	Embryonic organ development	29	0.023893925
GO:0035239	Tube morphogenesis	24	0.02400269
GO:0001655	Urogenital system development	22	0.024518911
GO:0010324	Membrane invagination	34	0.024948809
GO:0006897	Endocytosis	34	0.024948809
GO:0007596	Blood coagulation	21	0.025910578
GO:0050817	Coagulation	21	0.025910578
GO:0032535	Regulation of cellular component size	39	0.028157164
GO:0010557	Positive regulation of macromolecule biosynthetic process	74	0.028198513
GO:0009612	Response to mechanical stimulus	15	0.028898006
GO:0051174	Regulation of phosphorus metabolic process	59	0.029741894
GO:0019220	Regulation of phosphate metabolic process	59	0.029741894
GO:0045941	Positive regulation of transcription	66	0.030884384
GO:0009628	Response to abiotic stimulus	48	0.036186803
GO:0042325	Regulation of phosphorylation	57	0.036382059
GO:0001822	Kidney development	20	0.036765142
GO:0060429	Epithelium development	34	0.048127599
GO:0033273	Response to vitamin	16	0.049663031
GO:0051272	Positive regulation of cell motion	20	0.049846423
GO:0040017	Positive regulation of locomotion	20	0.049846423
Cellular component			
GO:0044459	Plasma membrane part	248	1.80E−19
GO:0005887	Integral to plasma membrane	152	5.18E−15
GO:0031226	Intrinsic to plasma membrane	154	7.05E−15
GO:0005886	Plasma membrane	344	3.22E−13
GO:0044421	Extracellular region part	117	3.93E−09
GO:0005615	Extracellular space	87	5.46E−07
GO:0000267	Cell fraction	110	5.86E−04
GO:0005578	Proteinaceous extracellular matrix	45	0.001110725
GO:0031988	Membrane-bounded vesicle	67	0.001230546
GO:0005626	Insoluble fraction	89	0.001617761
GO:0016023	Cytoplasmic membrane-bounded vesicle	64	0.003616814
GO:0005624	Membrane fraction	85	0.004330333
GO:0031982	Vesicle	73	0.006979539
GO:0031012	Extracellular matrix	45	0.008421052
GO:0044433	Cytoplasmic vesicle part	29	0.023217261
GO:0005829	Cytosol	121	0.035655389
GO:0031410	Cytoplasmic vesicle	68	0.039523539
GO:0045121	Membrane raft	24	0.041241162
GO:0015629	Actin cytoskeleton	36	0.049212125
Molecular function			
GO:0019838	Growth factor binding	26	3.29E−05
GO:0005509	Calcium ion binding	103	4.30E−04
GO:0043565	Sequence-specific DNA binding	70	0.01890116
GO:0046983	Protein dimerization activity	63	0.043858421

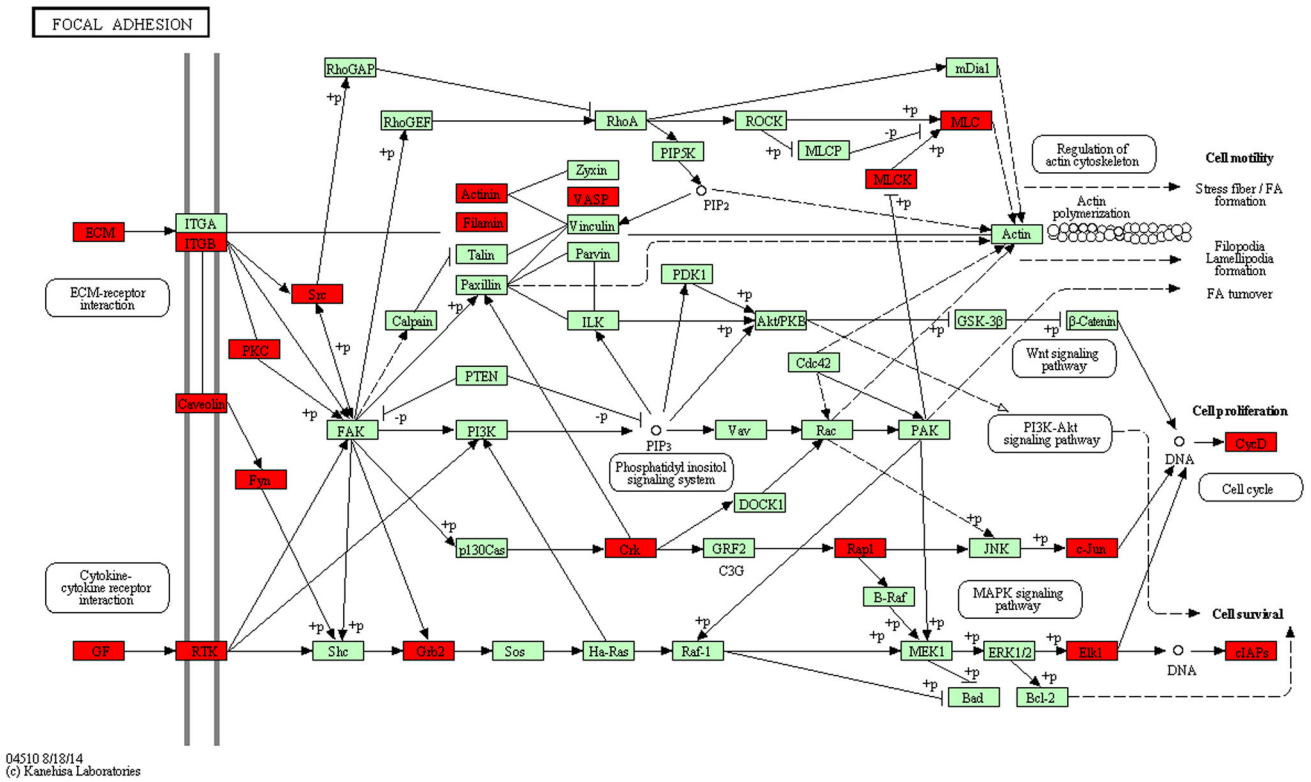

Fig. 1 The enriched KEGG pathway of DEGs (focal adhesion pathway). The *red icons* mean DEGs identified in this study

profiling of up- and down-regulated genes in each sample is shown in Fig. 3. The average fold changes of the up-regulated genes MMP12, COL11A1, THBS2, ADAM12, and FAP were 6.6-, 17.76-, 4.46-, 4.69-, and 2.77-folds, respectively. MMP12 and COL11A1 were dramatically up-regulated in three of five samples, while the other three genes were mild up-regulated in most of the NSCLC samples. The average fold changes of the down-regulated genes FABP4, CDH5, CAV1, TCF21, and ADRB2 were 24.9-, 3.5-, 18.28-, 8.3-, and 3.4-folds. FABP4 and CAV1 were dramatically down-regulated in four of five patient samples.

Discussion

Lung cancer is still the leading cause of cancer-related mortality all over the word [13]. Although there is a huge development in molecular techniques and lung cancer biology, many of the genetic alterations related to lung carcinogenesis still remain unknown. Microarray analysis can discover the expression alteration of a large number of genes simultaneously within tumors, which may help discover new signaling pathways or molecular mechanisms associated with tumorigenesis. In this study, we combined 15 microarray data sets to detect DEGs. We also performed GO term, KEGG, and PPI analysis of the DEGs and detected some important molecules and signaling pathways which may extend our understanding of the pathology of NSCLC and further guide the development of new therapeutic targets.

In this study, we found 1063 DEGs between NSCLC and NC tissues. GO analysis showed that 266 DEGs were enriched in cell proliferation regulation and 96 DEGs were enriched in DNA binding and growth factor binding. The results indicated that cell proliferation was dysregulated in NSCLC tissues. The fundamental abnormality of cancer cells was the continual unregulated cell proliferation [14], and numerous studies had proved the important role of EGFR in NSCLC, indicating that the GO analysis in this study was reasonable.

KEGG pathway analysis revealed that the DEGs between NSCLC and normal tissues are enriched in focal adhesion signaling pathways. Focal adhesions are the contact sites between the cytoskeleton and extracellular matrix through transmembrane proteins, integrins [15]. Cells received signals from extracellular microenvironment through focal adhesions to maintain proper cell survival, proliferation, differentiation, and motility through integrin-related signaling pathways [16–18]. The loss of the tight regulation of focal adhesions can lead to cancer progression and metastasis [19–21]. PPI network analysis for the top 20 significantly DEGs indicated that the significant hub

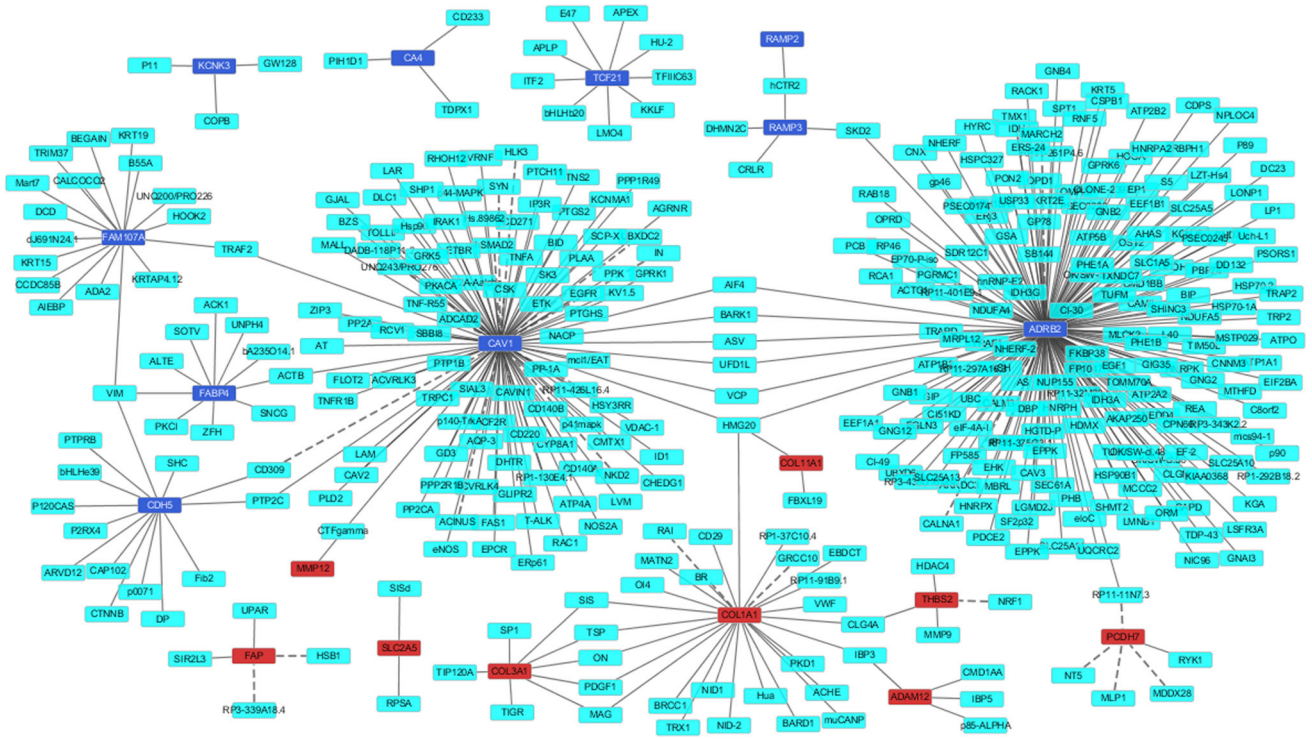

Fig. 2 PPI networks of the top ten most significantly up- or down-regulated DEGs. *Nodes* and *edges* represent proteins and interactions between proteins. The up-regulated genes in NSCLC were marked with *red* color and the down-regulated genes in NSCLC were marked with *deep blue* color

proteins were CAV1 and COL1A1, which are the important components of focal adhesion [21]. Our data indicated that focal adhesion components and related signaling pathways may play important roles in pathology of NSCLC and may shed light on discovery of new therapeutic targets of NSCLC.

In order to validate our meta-analysis data, we performed qRT-PCR to detect the expression of the top ten significant DEGs in NSCLC and NC tissues. We found that the expression patterns of the selected ten DEGs, including MMP12, COL11A1, THBS2, ADAM12, FAP, FABP4, CDH5, CAV1, TCF21, and ADRB2, were consistent with our meta-analysis and previous reports.

qRT-PCR results show that the mRNA levels of MMP12, COL11A1, THBS2, ADAM12, and FAP are significantly higher in NSCLC tissues than those in NC tissues. MMP12 is a 22 kDa metal-dependent proteinase which can degrade elastin, type IV collagen, fibronectin, laminin, gelatin, vitronectin, entactin, heparin, and chondroitin [22]. Many of the MMP12 substrates, such as collagen, laminin, and fibronectin, are important extracellular matrix molecules which can regulate cell shape, migration, and survival through focal adhesion [23]. Previous studies also reveal that MMP12 is correlated with early cancer-related deaths in NSCLC, especially for the patients who exposed to tobacco cigarette smoke [24].

Both COL11A1 and THBS2 participate in focal adhesion signaling pathways. COL11A1 is linked to ovarian cancer recurrence and poor survival. The invasion ability and oncogenic potential of ovarian cancer cells are suppressed by COL11A1 knockdown [25]. COL11A1 and THBS2 are overexpressed in lung cancer and can be recognized as a marker of lung cancer [26–28]. FAP is a serine protease selectively and highly expressed on the surface of cancer-associated fibroblasts, and it is important in the progression and prognosis in diverse malignancies [29]. The expression level of FAP is closely associated with tumor recurrence and poor clinical outcome in rectal and pancreatic cancer [30, 31]. However, the functions of FAP in NSCLC are poorly understood. One study reports that FAP is highly expressed in lung cancer stroma, and its high expression is a predictor of poor survival of NSCLC patients [32].

The expression of CAV1 and ADRB2 is lower in NSCLC tissues than that in NC tissues, and they are also the significant hub proteins of the PPI network in our study. Furthermore, CAV1 is involved in the focal adhesion signaling pathway. CAV1 is a major structural component of caveolae which is a specialized plasma membrane invagination [33]. The function of CAV1 in tumorigenesis is depending on tumor type and tumor stage. The expression of CAV1 is down-regulated in tumor cells and tissues isolated

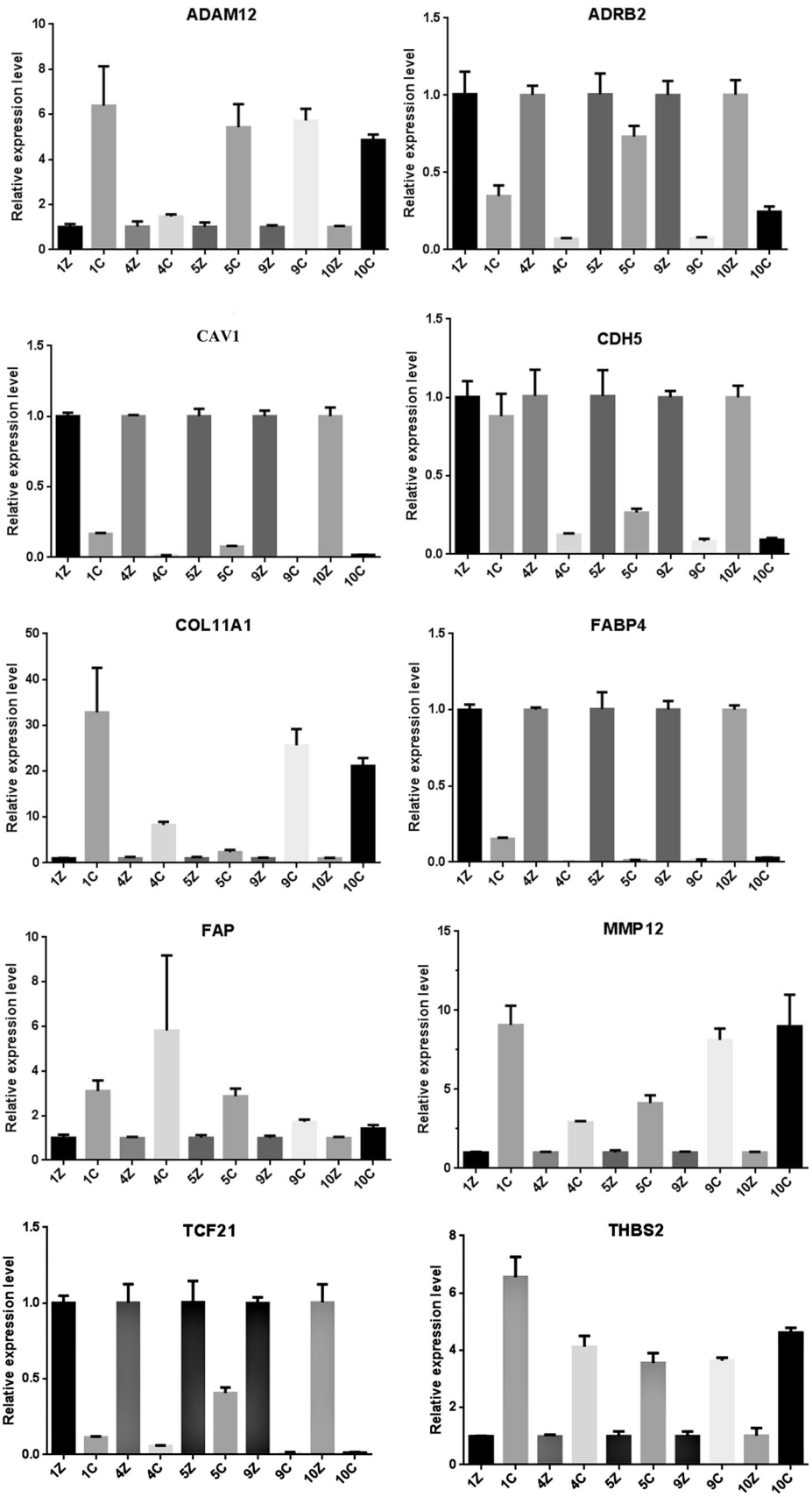

Fig. 3 qRT-PCR validation of the top ten most significantly up- or down-regulated DEGs in five pairs of NSCLC and NC tissues. ACTIN was used as an internal reference gene for normalization. The *graph* showed the relative expression levels of ADAM12, ADRB2, CAV1, CDH5, COL11A1, FABP4, FAP, MMP12, TCF21, and THBS2 between each pair of NSCLC and NC tissues. *Bar* graph represents mean ± SEM. Z means normal control tissues; *C* means non-small-cell lung cancer tissues

from breast, cervix, lung, and ovary [34–38], indicating that CAV1 may act as a tumor suppressor. Currently, the in vivo function of ADRB2 in NSCLC is largely unknown.

In summary, we used a meta-analysis approach to integrate 15 microarray data sets of NSCLC and identified DEGs and their biological function. We used qRT-PCR to validate the meta-analysis approach by detecting the expression of top ten significantly DEGs. Our study suggested that some DEGs, including MMP12, COL11A1, THBS2, FAP, and CAV1, might participate in the pathology of NSCLC and they might be potential therapeutic targets.

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Conflict of interest The authors declare that they have no conflict of interest.

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