Profiling of transcripts and proteins modulated by K-*ras* oncogene in the lung tissues of K-*ras* transgenic mice by omics approaches

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Abstract. The mutated K-*ras* gene is involved in ~30% of human cancers. In order to search for K-*ras* oncogene-induced modulators in lung tissues of K-*ras* transgenic mice, we performed microarray and proteomics (LC/ESI-MS/MS) analysis. Genes (RAB27b RAS family, IL-1RA, IL-33, chemokine ligand 6, epiregulin, EGF-like domain and cathepsin) related to cancer development (Wnt signaling pathway) and inflammation (chemokine/cytokine signaling pathway, Toll receptor signaling) were up-regulated while genes (troponin, tropomodulin 2, endothelial lipase, FGFR4, integrin α8 and adenylate cyclase 8) related to the tumor suppression such as p53 pathway, TGF-ß signaling pathway and cadherin signaling pathway were down-regulated by K-*ras* oncogene. Proteomics approach revealed that up-regulated proteins in lung adenomas of K-*ras* mice were classified as follows: proteins related to the metabolism/catabolism (increased from 7 to 22% by K-*ras* gene), proteins related to translation/transcription and nucleotide (from 4 to 6%), proteins related to signal transduction (from 3 to 5%), proteins related to phosphorylation (from 1 to 2%). ATP synthase, Ras oncogene family, cytochrome c oxidase, flavoprotein, TEF 1, adipoprotein A-1 BP, glutathione oxidase, fatty acid BP 4, diaphorase 1, MAPK4 and transgelin were up-regulated by K-*ras* oncogene. However, integrin α1, Ras-interacting protein (Rain), endothelin-converting enzyme-1d and splicing factor 3b were down-regulated. These studies suggest that genes related to cancer development and inflammation were up-regulated while genes related to the tumor suppression were down-regulated by K-*ras*, resulting in the tumor growth. Putative biomarkers such as cell cycle related genes (Cdc37), cancer cell adhesion (Glycam 1, integrin α8, integrin αX and Clec4n), signal transduction (Tlr2, IL-33, and Ccbp2), migration (Ccr1, Ccl6, and diaphorase 1 (Cyb5r3) and cancer development (epiregulin) can be useful for diagnosis and as prognosis markers and some of the target molecules can be applied for prevention of cancer.

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Introduction

Lung cancer is the leading cause of cancer deaths worldwide (1). Mutations of K-*ras* gene have been demonstrated in 40-50% of lung cancer and activating mutations of the K-*ras* oncogene are found in one-quarter to one-half of human lung adenocarcinomas. K-*ras* is a member of the Ras family, the most common oncogenes activated in a wide range of human cancers. Research on Ras oncogene has established mutations of three members of Ras gene family in various human cancers of tissue and organs, including urinary bladder, mammary gland, rectum, kidney, liver, lung, ovary, pancreas, stomach and the hematopoietic system. Ras proteins are activated by diverse extracellular signals, for example, via integrins or growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) that bind to their respective receptors with tyrosine-kinase activity, or by cytokines such as interleukin-2. Activated Ras proteins then pass the stimulating signal downstream via multiple effectors that can interact synergistically (2). Also, K-*ras* is a membrane-

associated GTPase signaling protein that regulates proliferation, differentiation and cell survival (3). The frequency of K-*ras* mutation has been demonstrated to be considerably high in certain types of cancers (4,5), such as in 75% of pancreatic tumors (6,7), 45% of colorectal cancers (7) and 48% of lung cancer (8). Some studies have proposed that K-*ras* mutations play a significant role in the onset and progression of lung cancer (9). In the mouse, K-*ras* mutations are found in >90% of spontaneous and chemically-induced lung tumors (10). Currently, based on the results of molecular analysis of spontaneous and chemically-induced tumors in K-*ras*, the mutation and overexpression of the transgene are considered to be the most probable mechanisms of enhanced carcinogenesis (11-13). Oncogenic K-*ras* is locked in an active form and is thus capable of activating downstream signaling cascades in the absence of external stimuli. K-*ras* activates effectors including the kinases Raf, PI3K, and MEKK, the GTPase Ral, and the signaling molecule AF6, which acts on gap junctions. K-*ras* signal transduction mediated by binding of growth factor, cytokines, or integrin also has inter-connections with other major players in tumorigenesis, such as the Wnt signaling cascade (via ß-catenin destruction complex) and the tumor suppressor p53 (14-16). In microarray and proteomics analyses, we confirmed that several genes and proteins related to cell growth, migration, cancer development, apoptosis, signaling pathway were up- and down-regulated by K-*ras* oncogene. To confirm induction and development of cancer by mutants, we generated the Lox-Stop-Lox K-*ras*^{G12D} Tg mice, in which expression of oncogenic K-*ras* is controlled by a removable transcriptional termination Stop element (17). In this study, the focus was on the survey of genes and proteins regulated by K-*ras* in lung tissues of K-*ras* transgenic mice. For this reason, K-*ras*^{G12D} mouse was generated and the following experiments were performed as described in the Materials and methods section.

Materials and methods

Generation of K-ras transgenic mice and genotyping. K-*ras*^{G12D} Tg mice were established by the method of DNA micro-injection into embryos of BDF1 mice, as previously described (18). Mutant K-*ras*^{G12D} gene expression vector carrying human surfactant protein C (SPC) promoter was used in order to regulate mutant K-*ras* gene to be expressed in the lung. These mice were obtained for modulator analysis by K-*ras* mutant in mouse model of lung cancer. Genomic DNAs were extracted from Tg mice tails and PCR analysis was performed to analyze K-*ras* gene expression. The cycling conditions were as follows: 94˚C, 5 min followed by 30 cycles of 94˚C, 30 sec; 60˚C, 30 sec; 72˚C, 30 sec and 72˚C 10 min in Takara PCR thermal cycler.

Histopathological examination. Tissue samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin/eosin according to standard methods. The histopathological diagnoses were based on the criteria described by Frith and Ward (19).

Microarray analysis and hybridization. Gene expression analysis was conducted on the RNA samples from the lung tissues of K-*ras* Tg mice. Labeling and hybridization were performed by instruction of Affymetrix 430 2.0 array chips (Affymetrix, Santa Clara, CA). Hybridization was performed at 55˚C for 16 h. After washing, the slide was dried by centrifugation at 800 rpm for 3 min at RT. Hybridization images on the slides were scanned by Applied Biosystems 1700 Chemiluminescent Microarray Analyzer (Applied Biosystems, CA).

Proteomics analysis. Proteins were loaded on 4-20% Tris-Glycine Gel (Invitrogen, Carlsbad, CA). The gel pieces were excised in 10 bands according to molecular weight and digested with sequencing grade modified trypsin (Promega, Madison, WI) at 12.5 ng/μl in 50 mM NH_4HCO_3 buffer (pH 8.0) at 37˚C for overnight. The resulting tryptic peptides were loaded onto a fused silica microcapillary column (15 cm x 75 μm) packed with C18 (5 μm, 200 Å) reversed phase resin and were separated by LC using a linear gradient of 5-50% buffer A in 65 min followed by 50-90% buffer B in 5 min (buffer A: 0.1% formic acid in H_2O, buffer B: 0.1% formic acid in acetonitrile) at a flow rate of 250 nl/min. The column was connected directly to LTQ ion-trap mass spectrometry (Thermo Finnigan, Waltham, MA) equipped with a nano-electrospray ion source and the eluent peptides were dynamically selected for fragmentation by the operating software. Protein identification was performed by using Spectrum Mill Proteomics Workbench Version A.03.03 (Agilent Technologies). Data files were extracted using Spectrum Mill Data Extractor. Searches were carried out against the human NCBInr database in both forward and reverse directions using the Spectrum Mill program with the following parameters: specific to trypsin with two missed cleavage; ±2.5 Da precursor-ion tolerance; and ±0.7 Da fragment-ion tolerance. Carboxyamido-methylation of cysteine and oxidation of methionines were allowed as variable modifications. The initial results were auto-validated using the following parameters for details on the protein mode: SPI (scored percent intensity) >70% for matches with score >7 for +1, >9 for +1, >9 for +3, >8 for +4; and SPI >90% for score >6 on +1. The proteins were summarized by validated peptides using the following parameters: >13 for protein score; SPI >70% for score >9 on peptide; and each protein included at least 2 distinct peptide hits. A semiquantitative analysis of protein profile data was performed by comparing the total peptide intensity with the peptides of an identified protein. The total peptide intensity was obtained by summing up the peptide intensities of the peptide hits for the protein. False positive rates were calculated, as described previously (20).

RNA isolation and cDNA synthesis for Q-PCR analysis. Total RNAs were isolated from the lung tissue of K-*ras* oncogene transgenic mouse to measure the quantity of the various genes or proteins. The lungs of K-*ras* transgenic mouse and non-transgenic mouse were collected and lysed using both TRIzol (Invitrogen) and homogenizer machine. The mixture was transferred to a new e-tube and was grinded by 1 ml needle. The mixture was centrifuged at 12,000 rpm for 10 min to remove the tissue debris and the supernatants were transferred to a new e-tube. Chloroform (200 μl) was added into the mixture and vortexed and then incubated at room temperature

Table I. Primer pairs used for real-time qPCR analysis of gene expression.

Gene	Forward primer	Reverse primer
Ccr1	5'-CAGAAGCCTACCCCACAACT-3'	5'-CTAGGACATTGCCCACCACT-3'
Ccl6	5'-TTATCCTTGTGGCTGTCCTTG-3'	5'-CATGGGATCTGTGTGGCATA-3'
IL-33	5'-TGGCCTCACCATAAGAAAGG-3'	5'-GGATACTGCCAAGCAAGGAT-3'
Itgax	5'-TGGCTATCACACAGGCAACT-3'	5'-TGCACAGTAGGACCACAAGC-3'
Tlr2	5'-GGCTCTTCTGGATCTTGGTG-3'	5'-TGTGAGTCCGGAGGGAATAG-3'
Etfb	5'-GCTGGCTGAAAAGGAGAAAG-3'	5'-GTCACCTGAGAGGCGAATGT-3'
Nt5c	5'-ACCGAGGTCTTCATCTGCAC-3'	5'-CCACCGTCTTGTCCCTAGTC-3'
Cyb5r3	5'-CTGTGTCGAGCGATGATGAC-3'	5'-CGGAATTCAATGGTGTCTCC-3'
Eef1b2	5'-GAGTGTGTCCGAAGCATTCA-3'	5'-TCTTCCAGCATATCCGTTCC-3'
Prdx3	5'-GAAAGAATGGTGGTTTGGGC-3'	5'-TGCTTGACGACACCATTAGG-3'
Ereg	5'-GCAGTTATCAGCACAACCGT-3'	5'-CAAGCAGTAGCCGTCCATGT-3'
Add3	5'-TATTCAACACGCCCCGATGT-3'	5'-CTGGTAGTCATAGTAGGCGA-3'
Cdc37	5'-GAGGTAGAGGAGAAATGTGC-3'	5'-GGTGGTCAGCGGTCTTGATC-3'
Sdc1	5'-ATCTTTGCTGTGTGCCTGGT-3'	5'-TAGAACTCCTCCTGCTTGGT-3'
Anxa4	5'-CAGGATTCAACGCAACTGAA-3'	5'-GGTGCTCTTGTAGGCACTCC-3'
Clec4n	5'-AATACTGAAGCGGAGCAGAA-3'	5'-GCCAGAACCTGACATTTTGA-3'
Glycam1	5'-CTCCCACCAGCTACACCAGT-3'	5'-TCCTGGGCCTCTTGATTCTC-3'
Ccbp2	5'-TGTAGTGACTATGCCCTTCT-3'	5'-CCAGGTATTTGTCCAGGCTC-3'
Xrn1	5'-ATGGGAAACAACAGGAATGG-3'	5'-CAGCACATCAGGCACTCACT-3'
Fgfr4	5'-GCCTTTCCTACCAGTCAGC-3'	5'-GGAGCACAAGCAGAACCAGT-3'
Itga8	5'-GACACCACCAACAACAGGAA-3'	5'-TCTCCAGTGATACAAAGGGG-3'
Tcrb-V13	5'-CTTGCCTCTCTTGACCACAG-3'	5'-AGAGCCAGGGATGAAAAATG-3'
Adcy8	5'-CGCAAATCGGAGGTAGTGAT-3'	5'-TGCCAGTGAAAAAGCCTAGC-3'

for 10 min until the mixture was divided into two layers. The mixture was centrifuged at 12,000 rpm for 10 min to remove the nuclear protein mixture. The supernatants were transferred to a new e-tube and 500 μl isopropanol was added to condense the RNA pellet. The mixture was centrifuged at 12,000 rpm for 20 min and the supernatants were discarded and then 1 ml of 75% ethanol was added to wash the pellet. The mixture was centrifuged at 12,000 rpm for 20 min and the supernatants were discarded. The pellet was dissolved in 100 μl diethylpyrocarbonate (DEPC)-treated water (Welgene, Daegu, Korea). Total RNAs were cleaned by RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The concentration of RNAs was measured and 5 μg each RNA was used to synthesize cDNA. RNAs were boiled for 2 min to unfold the secondary structure of RNAs. Three micrograms of oligo dT was added and the mixture was incubated at 65˚C for 5 min. This step indicated that oligo dT was attached to poly A tail of RNAs. After incubation, 5 μl of 10X reverse transcription buffer (BioLabs, Ipswich, MA), reverse transcriptase (BioLabs), 250 μM dNTP and DEPC water were added into the previous mixture at 42˚C for 1 h.

Quantitative real-time PCR. Real-time PCR was performed with relative quantification protocol on a MiniOpticon™ real-time PCR system (Bio-Rad, Hercules, CA), using iQ™ SYBR®-Green Supermix (Bio-Rad) for amplification detection. The real-time SYBR-Green assay was performed in a 20 μl reaction mixture with 2 μl of 25X diluted cDNA product, containing 1X iQ SYBR-Green Supermix (Bio-Rad) and 200 nM each primer. The PCR amplification profile was as follows: initial denaturation at 95˚C for 5 min; 40 cycles at 95˚C for 20 sec, Tm of samples for 20 sec and 72˚C for 20 sec. Optical data were collected after the annealing step of each cycle. Following PCR, reaction products were incubated for 1 min at 95˚C and then at 55˚C. PCR products were melted between 55˚C and 95˚C, then the temperature was increased up to 95˚C in 0.2˚C increments, 2 sec per increment. Optical data were collected over the duration of the temperature drop, with a dramatic increase in fluorescence occurring when the strands re-anneal. This was done to ensure that only one PCR product was amplified per reaction. Relative expression of the RT-PCR products was determined using the ΔΔCt method. This method calculates relative expression using the equation: Fold induction = 2^[-ΔΔCt], where Ct is the threshold cycle, i.e. the cycle number at which the sample's relative fluorescence rises above the background fluorescence and ΔΔCt = [Ct gene of interest (unknown sample) - Ct GAPDH (unknown sample)] - [Ct gene of interest (calibrator sample) - Ct CGAPDH (calibrator sample)]. Primers were purchased from M-biotech (Seoul, Korea). Primer pairs were chosen to minimize dimerization of primer and to generate an amplicon between 130 and 150 bp. All target genes were normalized to the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), using primers (5'-ACCACAGTC CATGCCATCAC-3' as sense primer, 5'-TCCACCACCCTG TTGCTGTA-3' as antisense primer) and compared to normal mouse of the appropriate strain for relative expression values. One of the control samples, non-Tg mouse was chosen as the calibrator sample and used in each PCR. Each sample was run in triplicate and fold changes represent the ratio of K-*ras* Tg mice to control (non-Tg mice) expression values. The sequences of the primers are listed in Table I.

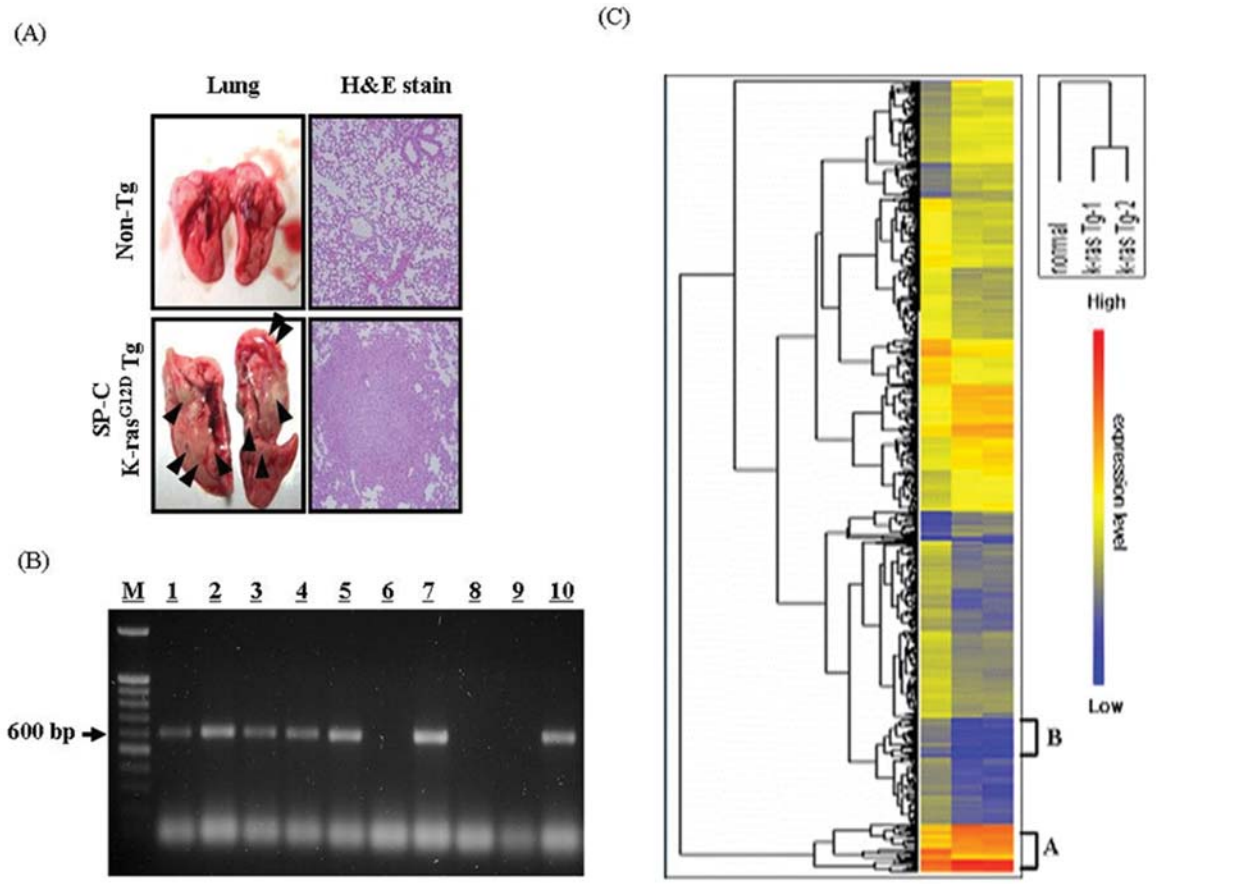

Figure 1. (A) Lung adenomas of K-*ras* Tg mouse. (B) Detection of K-*ras* gene expression by PCR in K-*ras* Tg mice. (C) Hierarchical clustering. Gene expression profiles of lungs of normal and K-*ras* Tg mice showing a distinct pattern. Most of the significantly up- and down-regulated genes are indicated in areas A and B, respectively.

Results

Establishment of K-ras Tg mouse model and histopathological examination. Histopathologically, several alveolar/bronchiolar hyperplasias and adenomas were observed in each lobe of the lung in the K-*ras* Tg mice (Fig. 1A). Adenomas were induced as early as week 4 in all K-*ras* Tg mice. The size of K-*ras* gene product of PCR using genomic DNA was ~600 bp as expected in the lanes 1, 2, 3, 4, 5, 7 and 10 (Fig. 1B) and these K-*ras*^{G12D} Tg mice were used for analysis of K-*ras* modulators in mouse model of lung cancer and such genes were classified based on molecular function. Gene expression profiles of normal lungs and adenomas showed a distinct pattern in hierarchical clustering. Most of the up-regulated genes were included in area A of the hierarchical clustering and most of down-regulated genes were included in area B (Fig. 1C).

Profiling analysis of genes modulated by K-ras oncogene. We classified up- and down-regulated genes with known functions based on molecular function (Fig. 2A) and pathway (Fig. 2B) involved in biological processes. With regard to up-regulated genes involved in molecular function, the genes were classified into receptor, nucleic acid binding, defense/immunity, oxidoreductase, kinase, hydrolase, etc. Also, down-regulated genes involved in molecular function were classified into cytoskeleton proteins, transcription factor, extracellular matrix, etc. The classification based on pathway of K-*ras* induced genes includes T-cell activation, toll receptor signaling, Wnt signaling, p53 pathway, inflammation mediated by chemokine and cytokine signaling, Alzheimer disease presenilin pathway, interleukin signaling, angiogenesis, apoptosis signaling pathway, etc. Down-regulated genes involved in pathway function were classified into inflammation mediated by chemokine and cytokine signaling, interleukin signaling, angiogenesis, Wnt signaling, TGF-ß signaling pathway, endothelin, PDGF signaling pathway, cadherin signaling pathway, etc. Many genes, of which expression are considered to be regulated by K-*ras*, were found in their expression pattern obtained from microarray in a highly consistent manner of K-*ras* regulation and 40 and 31 genes up- and down-regulated by K-*ras* were selected for quantitative real-time PCR analysis (Table II).

Profiling analysis of protein modulated by K-ras oncogene. Proteins modulated by K-*ras* oncogene were analyzed by LC-ESI-MS/MS. Total 653 proteins up-regulated and 263 proteins down-regulated by K-*ras* were classified by fold change, as shown in Table III. Proteins up- and down-regulated by K-*ras* in lung tissue in K-*ras* Tg mice are listed in Table IV. Proteins up- and down-regulated by K-*ras* oncogene were annotated by categories based on the GO (http://www.geneontology.org/): biological process using the house-made FindGo program as shown in Fig. 3. With regard to both up- and down-regulated proteins, the proteins were

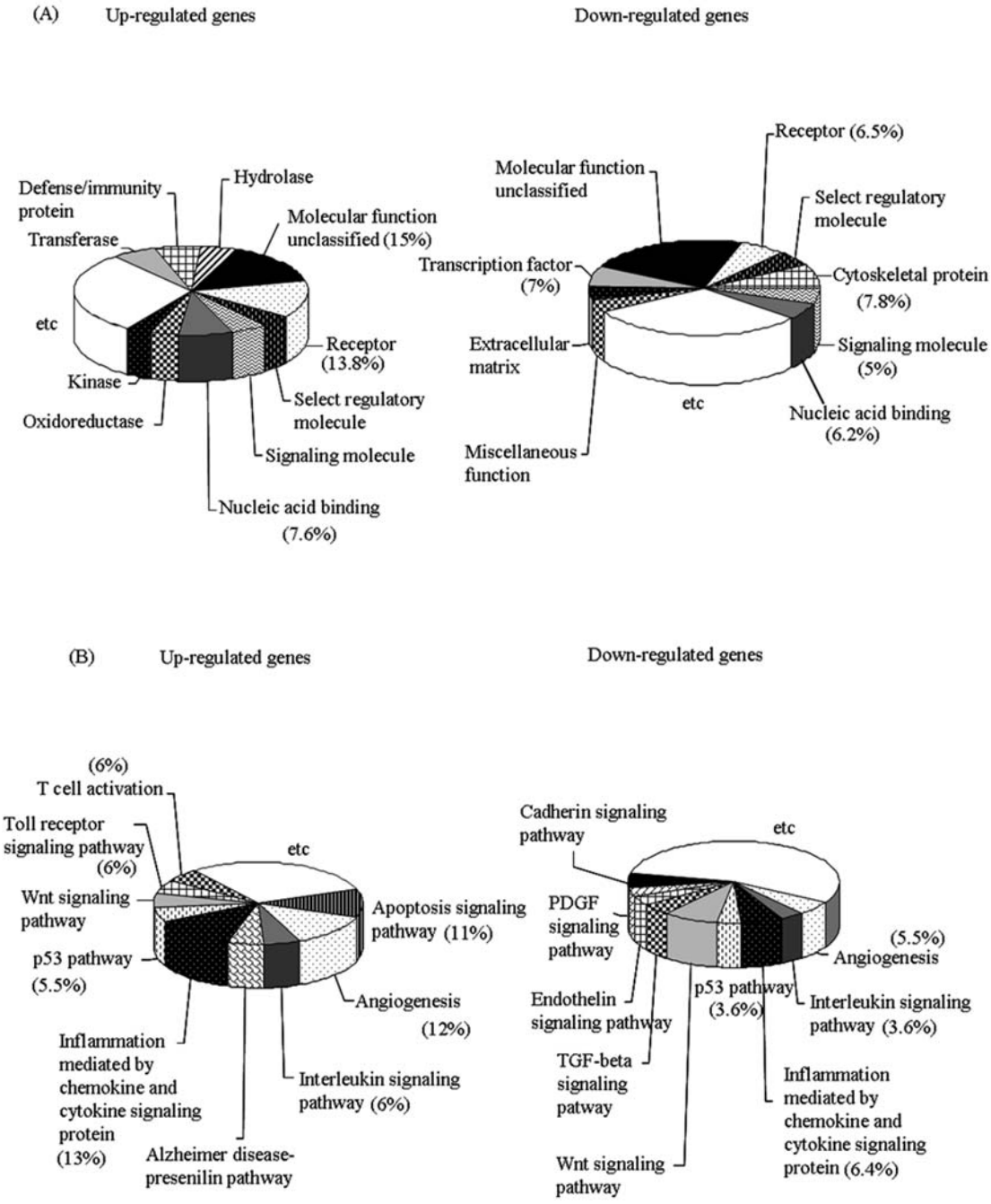

Figure 2. Pie charts showing gene classification based on molecular function (A) and pathway (B) of K-*ras* induced and down-regulated genes.

classified into proteolysis, cell adhesion, phosphorylation, development, cell cycle and cytokinesis, biological process, response, signal transduction, proliferation and apoptosis, translation and transcription and nucleotide, metabolism and catabolism, transport and localization, etc. Among the up-regulated proteins, the proportion of proteins involved in metabolism and catabolism was large.

Validation of differentially expressed genes by real-time qPCR. To verify the microarray analysis, we measured the expression levels of genes regulated by K-*ras* oncogene using real-time qPCR with 1X iQ SYBR-Green Supermix (Bio-Rad). Total RNAs (5 μg) were isolated from the lung tissues of normal and K-*ras* TG mice and 25X diluted cDNAs from total RNAs were used for real-time qPCR analysis. Non-TG mice were used as negative control in this experiment. The expression levels of epiregulin (Ereg), interleukin-33 (IL-33), integrin α X (ItgαX) and annexin A4 (Anxa4) were significantly increased in the lung tissues of K-*ras* TG mice (>10-fold). We also confirmed that expression of genes regulated by K-*ras* oncogene [chemokine (C-C motif) receptor 1 (Ccr1), chemokine (C-C motif) ligand 6 (Ccr6), toll-like receptor 2 (Tlr2), C-type lectin domain family 4, member n (Clec4n), and 5',3'-nucleotidase, cytosolic (Nt5c)] were increased. Syndecan 1 (Sdc1), Cdc37 and glycosylation-dependent cell adhesion molecule 1 (Glycam 1) were also increased 3.4-, 3.2- and 3.5-fold, respectively. Adducin 3 (Add3), electron transferring flavoprotein ß polypeptide (Etfb),

Table II. Up- and down-regulation of genes by K-*ras* oncogene.

A, Genes up-regulated by K-*ras* oncogene

Regulation by K-*ras*	Gene title	Fold change
Up	Epiregulin	2.50
	Chemokine (C-C motif) ligand 6	2.11
	Interleukin-33	1.86
	α fetoprotein	2.04
	Amphiregulin	2.41
	Membrane-spanning 4-domains, subfamily A, member 6D	2.32
	Serine/threonine kinase 39, STE20/SPS1 homolog (yeast)	2.31
	ATPase, H+ transporting, lysosomal V0 subunit D2	2.35
	S100 calcium binding protein G	1.94
	Tumor necrosis factor (ligand) superfamily, member 9	2.22
	Sorting nexin 6	1.81
	Integrin α X	1.70
	RAB27b, member RAS oncogene family	1.46
	Ras-related GTP binding D	1.32
	C-type lectin domain family 4, member n	1.81
	Colony stimulating factor 2 receptor, ß 1, low-affinity (granulocyte-macrophage)	1.89
	Glutaredoxin	1.47
	SH2 domain protein 1B1	1.42
	Ankyrin 3, epithelial	1.76
	Cathepsin H	1.43
	Cathepsin K	1.90
	Cathepsin C	1.21
	Purinergic receptor P2Y, G-protein coupled 13	1.41
	Syndecan 1	1.33
	Leukotriene C4 synthase	1.51
	Chemokine (C-C motif) receptor 1	1.27
	Chemokine (C-C motif) ligand 6	1.43
	Neurotrophin 5	1.30
	Toll-like receptor 2	1.66
	Interleukin 1 receptor antagonist	1.02
	Histocompatibility 2, class II, locus Dma	1.19
	HLA-B-associated transcript 1A	1.36
	Mannose receptor, C-type 1	1.37
	Protein arginine N-methyltransferase 8	1.65
	C-type lectin domain family 4, member a2 /// C-type lectin domain family 4, member b1	1.19
	Caspase recruitment domain family, member 11	1.38
	Cytochrome b-245, ß polypeptide	1.10
	Cytochrome c oxidase, subunit VI a, polypeptide 2	1.14
	Cadherin EGF LAG seven-pass G-type receptor 1	1.10
	EGF-like-domain, multiple	1.28

B, Genes down-regulated by K-*ras* oncogene

Regulation by K-*ras*	Gene title	Fold change
Down	Insulin-like growth factor binding protein 2	-2.86
	Contactin 1	-2.14
	Adrenomedullin	-2.08
	Collagen and calcium binding EGF domains 1	-1.95
	Dynein, axonemal, intermediate chain 2	-1.88
	Titin	1.65
	Latrophilin 3	-1.56
	Myeloid leukemia factor 1	-1.51

Table IIB. Continued.

Regulation by K-*ras*	Gene title	Fold change
Down		
	5'-3' exoribonuclease 1	-1.52
	Prostaglandin F receptor	-1.44
	Vav 3 oncogene	-1.39
	δ/notch-like EGF-related receptor	1.47
	Cytochrome P450, family 2, subfamily d, polypeptide 22	-1.34
	Tropomodulin 2	-1.20
	ATP synthase, H+ transporting, mitochondrial F0 complex, subunits	1.15
	Adenylate cyclase 8	-1.16
	RAB36, member RAS oncogene family	-1.30
	Tropomyosin 2	-1.31
	Calponin 3, acidic	-1.15
	Troponin I, cardiac	-1.20
	Lactotransferrin	-1.47
	Follistatin	-1.26
	Annexin A8	-1.19
	Troponin T2, cardiac	-1.14
	Chemokine binding protein 2	-1.11
	T-cell receptor ß, variable 13	-1.07
	Lipase, endothelial	-1.12
	Fibroblast growth factor receptor 4	-1.19
	Calcium channel, voltage-dependent, ß 2 subunit	-1.04
	Integrin α 8	-1.24
	Desmin	-1.17

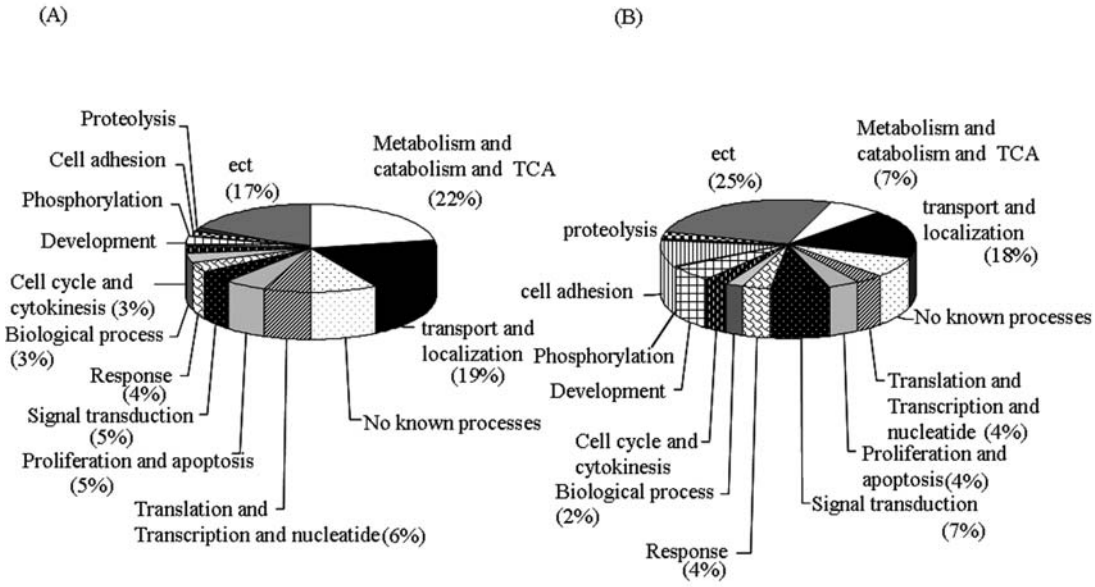

Figure 3. Pie charts showing classification of up-regulated proteins (A) and down-regulated proteins (B) in lung adenomas of K-*ras* Tg mice. Proteins up- and down-regulated by K-*ras* oncogene were annotated by categories based on the GO (http://www.geneontology.org/): biological function using the house-made FindGo program.

peroxiredoxin 3 (Prdx3), eukaryotic translation elongation factor 1 ß 2 (Eef1b2) and cytochrome b5 reductase 3 (Cyb5r3) were weakly increased (Fig. 4A). The expression levels of T-cell receptor ß V13 (Tcrb-V13), chemokine binding protein 2 (Ccbp2), 5'-3' exoribonuclease 1 (Xrn 1), integrin α8 (Itgα8), adenylate cyclase 8 (Adcy8), fibroblast growth factor receptor 4 (Fgfr4) were decreased by K-*ras* oncogene (Fig. 4B).

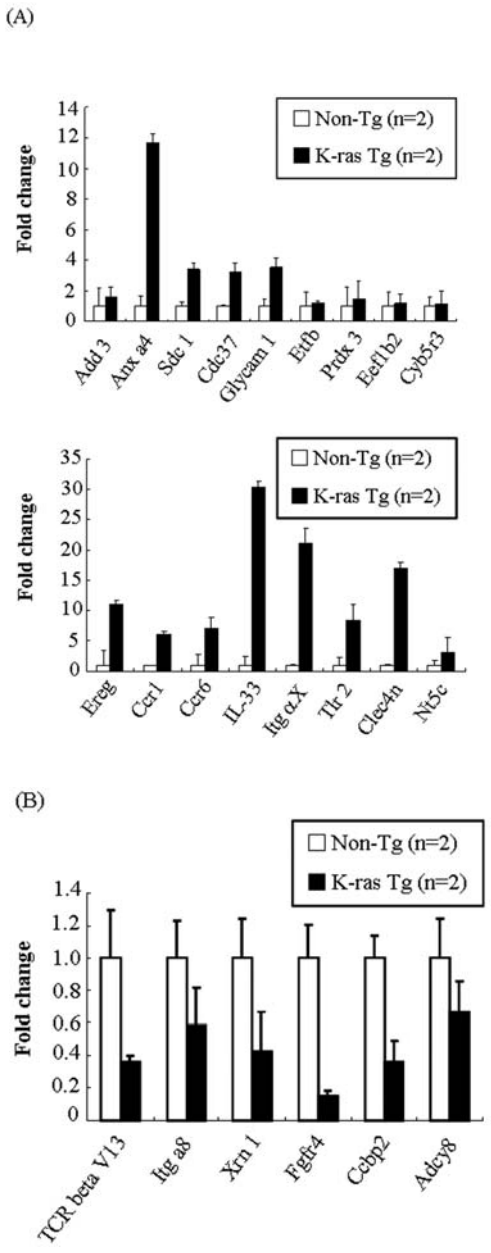

Figure 4. The expression level, which was obtained from Omics and real-time qPCR data of the selected as the candidates in the enhanced carcinogenesis in K-*ras* Tg mice. Genes up-regulated (A) and down-regulated (B) in lung adenomas of K-*ras* Tg mice. The gene expression level obtained from real-time qPCR was normalized by GAPDH. The respective fold changes were expressed by comparing with the values of non-Tg mice which were set as 1. Data are mean ± SD of two independent experiments, each performed in triplicates.

Table III. Proteomics analysis of protein profiling of lung proteins modulated by K-*ras* oncogene.

	Mus musculus DB	
Fold change	Up-regulated	Down-regulated
		48
<2	40	48
2-4	35	68
5-9	19	41
>10	559	79
Total	653	236

Data files were extracted using Spectrum Mill Data Extractor with the parameters of [MH^+] 600 to 40000 and minimum signal-to-noise (S/N) 25. Searches were carried out against the human NCBInr database in both forward and reverse directions using the Spectrum Mill program (Agilent Technologies) with the following parameters: specific to trypsin with two missed cleavage; ±2.5 Da precursor-ion tolerance; and ±0.7 Da fragment-ion tolerance. The initial results were auto-validated as described in the Materials and methods section.

Discussion

The *ras* proteins are pivotal regulators of cellular proliferation, differentiation, motility, and apoptosis (21). K-*ras* oncogenes are frequently detected in mouse lung tumors (22-24). Activating point mutations in the K-*ras* gene have been found in 30-50% of human lung adenocarcinomas and are believed to play a key role in this malignancy (25). To analyze profiling of genes and proteins regulated by K-*ras* oncogene in lung tissue of K-*ras* Tg mice, K-*ras*^{G12D} Tg mice, expressing mutant type K-*ras* gene in lung tissue by the regulation of SPC promoter sequence, were generated by the method of DNA microinjection into embryos of BDF1 mice as previously (18). K-*ras* Tg mice and non-Tg mice were confirmed by PCR genotyping with detection of only transgene-specific sequence using genomic DNA. The K-*ras* gene was identified in transgenic mice. Total RNAs from the lung tissues of K-*ras* Tg mice were isolated and cDNAs were synthesized from RNAs. cDNA was used to analyze genes by using Affymetrix chips and proteins modulated by K-*ras* oncogene were analyzed by MALDI-TOF. In microarray analysis, the genes involved in the molecular function (nucleic acid binding, receptor and signaling molecule, etc.) and pathway (angiogenesis, inflammation mediated by chemokine and cytokine signaling, and apoptosis, etc.) were up-regulated, and the expression of several genes for biological processes showed a decreasing tendency in K-*ras* Tg mice. Also, proteins involved in metabolism and catabolism, transport and localization, proliferation and apoptosis, cell cycle, development were up-regulated by K-*ras* oncogene, whereas proteins were down-regulated by K-*ras* in metabolism and catabolism and (from 22 to 7%), transport and localization (from 19 to 18%), proliferation and apoptosis (from 5 to 4%), translation and transcription and nucleotide (from 6 to 4%). Genes and proteins related to carcinogenesis or K-*ras* mediated lung cancer were selected and the expression level of these genes was confirmed by real-time qPCR. The genes showed a similar expression pattern to the results of Omics analysis in real-time qPCR. Epiregulin, IL-33, integrin αX, toll-like receptor 2, Clec4n and annexin A4 as candidate genes responsible for the enhanced carcinogenesis in K-*ras* Tg mice were significantly up-regulated by K-*ras* oncogene. Epiregulin, the newest member of the family of EGF-like ligands, is present in some normal human tissues and cancer cell lines. It may be involved in normal growth development and in cancer progression (26). Recent studies have suggested that it is overexpressed in human prostate, colon, pancreatic and lung cancers (27-30). Toll-like receptor 2, a membrane receptor found at the surface of immune system

Table IV. Up- and down-regulation of proteins by k-ras.

A, Proteins up-regulated by K-*ras*

Protein name	Accession No.	Peptides unique No.
ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d	21313679	59
ATP synthase, H+ transporting, mitochondrial F1F0 complex, subunit e	59808056	26
ATP synthase F0 subunit 8	34538602	23
RAB7, member Ras oncogene family	21263432	12
RAB11B, member Ras oncogene family	6679599	43
RAB2, member Ras oncogene family	6679583	26
RAB18, member Ras oncogene family	10946940	19
Cytochrome b-5	6755258	7
Ubiquinol-cytochrome c reductase complex protein isoform 1	13385268	41
Cytochrome c oxidase subunit IV isoform 1	37574048	26
Cytochrome b5 outer mitochondrial membrane precursor	6753498	20
Cytochrome c oxidase, subunit VIb polypeptide 1	31542438	19
Cytochrome c oxidase, subunit Vb	13385090	11
Cytochrome P450, family 2, subfamily b, polypeptide 10 isoform 1	112807195	9
UMP-CMP kinase	6753576	7
Proteasome activator subunit 2 isoform 1	23821758	35
Glutathione peroxidase	20137004	34
Glutathione peroxidase 3 precursor	2673845	18
Glutathione peroxidase 4 isoform 1 precursor	1170039	12
Microsomal glutathione S-transferase 1	90903233	10
Fatty acid binding protein 3, muscle and heart	14318725	10
Fatty acid binding protein 4, adipocyte	6753810	10
Ras suppressor protein 1 (Rsu-1) (RSP-1)	548879	18
Related RAS viral (r-ras) oncogene homolog 2		13
k-ras cellular oncogene	13399308	19
Ras suppressor protein 1 (Rsu-1) (RSP-1)	1213018	19
Voltage-dependent anion-selective channel protein 1	10720404	32
Voltage-dependent anion channel 2	6755965	15
Electron transfer flavoprotein subunit		
α, mitochondrial precursor	21759113	29
Electron transferring flavoprotein, ß polypeptide	38142460	25
Heat shock protein 1 (chaperonin)	31981679	27
Heat-shock protein ß-1	547679	17
Dodecenoyl-Coenzyme A δ isomerase		
(3,2 trans-enoyl-Coenyme A isomerase)	31981810	23
Aacetyl-Coenzyme A acetyltransferase 3	110625948	8
Peroxiredoxin 5 precursor	6755114	19
Peroxiredoxin 3	6680690	8
Thioredoxin 1	6755911	22
Eukaryotic translation elongation factor 1 γ	110625979	18
Eukaryotic translation elongation factor 1 ß 2	24586721	10
Eukaryotic translation elongation factor 1 δ isoform a	56699438	9
Vesicle-associated membrane protein, associated protein A	94721328	17
SEC22 vesicle trafficking protein-like 1	6755448	12
Histocompatibility 2, class II antigen A, ß 1	46358078	7
H-2 class I histocompatibility antigen,		
D-B α chain precursor [H-2D(B)] HLA-B-associated transcript 1A	122122	7
		13
Histocompatibility 2, class II antigen A, ß 1	9790069	7

Table IVA. Continued.

Protein name	Accession No.	Peptides unique No.
Cell division cycle 37 homolog	7949018	5
Apolipoprotein A-I binding protein	21553309	4
Adducin 3 (γ)	31542111	2
Ribosomal protein, large P2	83745120	59
Chloride intracellular channel 5	27369886	32
Diaphorase 1 (cytochrome b5 reductase 3)	19745150	32
Tumor-associated calcium signal transducer 1	112293275	5
Annexin A4	33416530	28
Tropomyosin 4	47894398	26
NADH dehydrogenase (ubiquinone) 1 α subcomplex, 4	33563266	26
6-phosphogluconolactonase	13384778	25
Transgelin	6755714	24
Guanine nucleotide-binding protein G(i)	1730229	22
Transmembrane emp24-like trafficking protein 10 (yeast)	40555903	20
SH3 domain binding glutamic acid-rich protein-like 3	18017602	20
Calpain small subunit 1	18202239	19
S100 calcium binding protein G	14789635	15
Septin 2	6754816	14
Gelsolin-like capping protein	110227377	14
Sideroflexin 3	16716499	14
Lactate dehydrogenase 2, B chain	6678674	13
Mitogen activated protein kinase 3	21489933	13
Prenylcysteine oxidase 1	13385294	11
Sulfide:quinone oxidoreductase, mitochondrial precursor	27151701	9
Galactokinase 1	29747831	6
Glycosylation	33468855	6

B, Proteins down-regulated by K-*ras*

Protein name	Accession No.	Peptides unique No.
Procollagen, type VI, α 2	22203747	6
Complement component 3	28175786	5
Pregnancy zone protein	34785996	3
Integrin α 1	84370023	3
Ras-interacting protein 1 (Rain)	81174999	2
GAG12	29420429	2
Splicing factor 3b, subunit 3	19527174	2
Endothelin-converting enzyme 1d	67810530	2

cells, plays a role in signal transduction, immune response, induction of apoptosis (31,32). It was reported that TLR2 might be used for immunotherapy of malignant tumors (33). IL-33 and Clec4n were involved in immune response and have cytokine and receptor activity, respectively. Clec4n has diverse functions, such as cell adhesion, signaling between cells (34,35). The expression levels of syndecan 1, chemokine (C-C motif) receptor 1, chemokine (C-C motif) ligand 6, cdc37 and glycam 1 were increased in K-*ras* Tg-mice. Syndecan, a cell surface proteoglycan, exhibits a molecular polymorphism during lung development (36). Syndecan 1 might be involved in pathogenesis of human lung cancer and non-small cell lung carcinoma (37,38). Chemokine (C-C motif) receptor 1 and chemokine (C-C motif) ligand 6, which have an important role in the recruitment of leukocytes to the site of inflammation, promote tumorigenesis and metastasis, respectively (39). The migration and metastasis of tumor cells share many similarities with leukocyte trafficking, which is mainly regulated by chemokine receptor-ligand interactions (40). Glycam 1, a member of the glycoprotein mucin family and integrin αX are involved in the cell adhesion and signal transduction (41,42). Cdc37, an essential component of the sevenless receptor/mitogen-activated kinase protein (MAPK) signaling pathway, is involved in cell division cycle (43). The expression levels of Prdx3, Etfb, Eef1b2, Nt5c, and Cyb5r3 were weakly or negligibly increased by K-*ras*

oncogene. Whereas, TCRb-V13, Ccbp2, Xrn 1, Itgα8, Adcy8 and Fgfr4 were down-regulated in lung tissue of K-*ras* Tg mice. TCRb-V13 regulates cellular defense response and has receptor activity. Xrn1 is involved in 5' to 3' RNA degradation. This enzyme has also been identified as a candidate tumor suppressor gene in osteogenic sarcoma (44). Integrin α8 is known to regulate cell adhesion, migration, and proliferation. Adhesion of cells to extracellular matrix via integrins is necessary to enable migration (45). CCBP2 gene encodes a ß chemokine receptor. Chemokines and their receptor-mediated signal transduction are critical for the recruitment of effector immune cells to the inflammation site (46). A specific role for FGFR4 is not well established in cancer, but altered expression has been documented in breast, lung, pancreatic and prostate cancers (47). The FGF/FGFR receptor (FGFR) signaling pathway plays a pivotal role in cellular biology, being involved in differentiation, angiogenesis and motility (48-50). Therefore, genes and proteins up- and down-regulated by K-*ras* oncogene are considered to be important for tumorigenesis in K-*ras* Tg mice.

In conclusion, our results suggest that overexpression of transgene plays an important role in the carcinogenesis in K-*ras* mice. Genes and proteins related to carcinogenesis or K-*ras* mediated lung cancer are considered to be the candidate genes responsible for the enhanced carcinogenesis in the K-*ras* Tg mice.

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