

Differential Expression of Gene Profiles in MRGX-treated Lung Cancer

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Key Words

gene ontology, lung cancer, microarray, modified regular ginseng extract (MRGX)

Abstract

Objectives: Modified regular ginseng extract (MRGX) has stronger anti-cancer activity-possessing ginsenoside profiles.

Methods: To investigate changes in gene expression in the MRGX-treated lung cancer cells (A549), we examined genomic data with cDNA microarray results. After completing the gene-ontology-based analysis, we grouped the genes into up- and down-regulated profiles and into ontology-related regulated genes and proteins through their interaction network.

Results: One hundred nine proteins that were up- and down-regulated by MRGX were queried by using IPA. IL8, MMP7 and PLAUR and were found to play a major role in the anti-cancer activity in MRGX-treated lung cancer cells. These results were validated using a Western blot analysis and a semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis.

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Conclusions: Most MRGX-responsive genes are up-regulated transiently in A549 cells, but down-regulated in a sustained manner in lung cancer cells.

1. Introduction

Ginseng has been most widely used as herbal medicine in Eastern Asian countries, including Korea, China, and Japan. Ginseng is a species of a perennial plant belonging to the genus *Panax* in the family *Araliaceae*, and the ginseng species are only found in the northern hemisphere from Siberia to Vietnam. In general, Asian ginseng (*Panax ginseng*) and American ginseng (*Panax quinquefolius*) are most well-characterized, and these ginseng ginsenosides have been quantitatively and qualitatively characterized by ginsenoside profiling [1]. In particular, *P. ginseng* contains more than thirty pharmacologically-valuable ginsenosides [2]. Among them, ginsenosides such as Rg3, Rh2, Rb2, Compound K, and Rf2 are known to possess anti-cancer activities against colorectal cancer, colon cancer, hepatoma and breast cancer [3-6]. The ginsenosides exhibit anti-cancer activity by directly inhibiting cancer cell growth and metastasis *in vivo* and *in vitro* [7]. The major ginsenosides, such as Rb1, Rb2 and Rg1, induce apoptosis of

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lung cancer via an extrinsic apoptotic pathway [8]. These results suggest that specific ginsenosides play critical roles in the inhibition of cancer cell proliferation.

Lung cancer, comprising of a majority of non-small-cell lung carcinomas (NSCLCs) of up to 85%, is the second leading cause of cancer death worldwide [9]. Thus, lung cancer is still considered as an extremely lethal cancer [10]. Surgical and combined therapies have contributed to reducing the mortality and the morbidity of lung cancer over last several decades; however, these therapies have limitations. Thus, complementary and alternative therapies with herbal medicine have recently been introduced in the Western medical society. However, few studies have reported on the anti-cancer activity of regular ginseng extract on a molecular biological level.

In this study, we prepared a modified regular ginseng extract (MRGX) that reinforced some ginsenosides from regular ginseng butanol-extract (GBX) by using treatments with enzymes such as laminarinase and pectinase for stronger anti-cancer effect. The assessments of the gene expression profiles in human lung cancer samples by using cDNA microarray technology showed that some genes were down-regulated while others were up-regulated during the MRGX-treatment process. Because gene expression might change during the lung cancer cell death process, such studies should use specific components and not serum that contains a mixture of ginsenosides.

2. Materials and Methods

2.1. Preparation of MRGX extract

The root of regular ginseng (4 yr old) was purchased from the National Agricultural Cooperative Foundation in Chuncheon-gnam-do, Korea. A total of 20 g of pulverized ginseng root powder was suspended in 380 ml of distilled water and then sterilized at 121 °C for 15 min. The ginseng butanol extract (GBX) contained compounds with specific anti-lung cancer activity. We used GBX as a control of regular ginseng. In addition, the suspension was treated with an aliquot of filter-sterilized commercial enzymes (laminarinase: 100 mg, pectinase: 100 mg) with equimolar ratio (1:1, specific activity unit); the mixture was then incubated at 40 °C for 2 days and evaporated to dryness at 60 °C. These enzymes-modified ginseng powders were suspended in 400 ml of 80% (v/v) methanol. The suspension was treated in an ultrasound bath for 5 min and filtered through Whatman No. 2 filter paper. The filtrates were combined and evaporated to dryness at 50 °C. The extract was diluted to a concentration of 10% (w/v) in 70% ethanol. In the present study, the finally-obtained sample was called the modified regular ginseng extract (MRGX).

2.2. Cell culture

Human lung cancer (A549) cells were obtained from American Type Culture Collection (Rockville, MD). Cell lines were grown in DMEM (Dulbecco Modified Eagle's Medium, Sigma, USA) supplemented with 10% (v/v) FBS (Fetal Bovine Serum, GIBCO, NY) and 1% (w/v) penicillin-streptomycin (GIBCO, NY). The cells were incubated at 37 °C with 5% (v/v) CO₂ for 24 h. The density of A549 cells was adjusted to 5×10^3 cells/well in a 96-well plate. After a 24-h incubation, the cells were treated with MRGX at 50 µg/ml concentrations. The appropriate dose was determined by evaluating the cytotoxicity of MRGX for 24 h. To the cell solution, 10 µl of cell-counting kit-8 solution (Dojindo, Japan) was added for 1 h. Cell viability was determined by using a microplate reader (Sunrise, Tecan, Switzerland) to measuring the absorbance at 450 nm.

2.3. Microarray analysis

For the microarray analysis of the MRGX-treated lung cancer cells, a human twin 44K cDNA chip was used for the transcription profiling analysis. Total RNA was extracted from vehicle- or MRGX-treated lung cancer cells, and cDNA probes were prepared by using reverse transcription of 50 mg of RNA in the presence of aminoallyl-dUTP, followed by coupling with Cy3 dye (vehicle) or Cy5 dye (MRGX-treated). The mixed Cy3- and Cy5-labeled RNA from lung cancer cells (A549) was hybridized with one side of the Twin Chip™ Human 44K (Genocheck, Seoul, Korea), and that from MRGX-treated cells was hybridized with the other side of the chip.

Fluorescent images were quantified and normalized, as described previously [11]. This experiment was repeated four times. Genes were considered to be differentially expressed when the global M value, $\log_2(R/G)$, exceeded |1.0| (twofold) with a *P*-value < 0.05 after a significance analysis of the microarray (SAM). A student's *t*-test was applied to assess the statistical significance of the differential expression of any gene between young and senescent HDFs (Human Dermal Fibroblasts) at each time point after MRGX treatment. To analyze the biological significance of the changes, we searched the gene ontology clone annotation (<http://www.geneontology.org>) and categorized the array data into specific gene groups. To verify the microarray data, we analyzed the total RNA extracted from cells by using a semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) and 2% agarose gel electrophoresis.

2.4. Ontology-related network analysis

To study the biological functions of ontology-related regulated genes and proteins through their interaction network, we conducted a bioinformatic protein network anal-

ysis by using an ingenuity pathways analysis (IPA, <http://www.ingenuity.com>). The IPA identifies the protein interaction network on the basis of a regularly-updated "Ingenuity Pathways Knowledge-base." The updatable database containing millions of individual protein-protein relationships was retrieved from the biological literature. Network generation was optimized to include as many proteins from the inputted expression profile as possible and aimed at the production of highly-connected networks.

2.5. Semi-quantitative RT-PCR confirmation

Total RNA was extracted from young and senescent cells by using an acid guanidinium thiocyanate phenol-chloroform extraction-based method. To compare the relative amounts of mRNA in young and senescent cells, we performed a semi-quantitative RT-PCR. A series of mixtures was prepared by mixing RNA from young and senescent cells as indicated, so that each mixture had the same total amount of RNA (2 µg) in a constant volume (12 µl). The RT reaction was carried out in a final volume of 20 µl by using Superscript II reverse transcriptase according to the manufacturer's protocol, and 4 µl of the final RT product mix was then PCR amplified. The primer sets used were 5'-ATCTGGCAACCCTAGTCTGC-3' and 5'-GTGCTTCCACATGTCCTCAC-3' for IL8, 5'-GACATCATGATTGGCTTTGC-3' and 5'-TCCTCATCGAAGTGAGCATC-3' for MMP7 and 5'-GTGAGGAAGCCCAAGCTACT-3' and 5'-ATGTCCAAGGTGGCTTCTTC-3' for PLAUR.

2.6. Western blot analysis

Expression levels of signaling proteins were examined by using Western blot analyses. In brief, 30 µg of denatured protein were run using 12% polyacrylamide gel electrophoresis and then transferred onto a nitrocellulose membrane. The transferred nitrocellulose membrane was stained with Ponceau S to position the proteins. The blotted membrane was blocked for 1 h with 5% (w/v) skimmed milk in TTBS (Tween-20 and Tris-buffered saline), followed by incubation with a dilution of primary antibodies (Cell Signal, Boston, USA), including interleukin-8 (IL-8), matrix metalloproteinase 7 (MMP7) and plasminogen activator, urokinase receptor (PLAUR), at room temperature for 2 h or at 4°C overnight. The membrane was washed three times for 5 min with 0.1% (v/v) TTBS before incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or HRP-conjugated rabbit anti-goat IgG with a 1:2000 dilution in TBS containing 5% (w/v) skimmed milk at room temperature for 1 h. The membrane was rinsed three times with TBS-0.1% (v/v) Tween-20 for 5 min. The Pierce® ECL system (Thermo Scientific, San Jose, CA) was used to develop the proteins on an X-ray film (Kodak, Seoul, Korea). The expression levels of proteins were quantitatively ana-

lyzed with GAPDH as an internal control.

3. Result

3.1. Analysis of MRGX related gene expression in lung cancer cells

To analyze the MRGX-related gene expression in lung cancer cells, we used a cDNA microarray analysis approach. Microarray data were filtered and combined using gene symbols; then, network analyses were performed. The clustered microarray data showed that groups of genes in lung cancer and MRGX-treated lung cancer cells were regulated differently by 50 mM of MRGX. Genes with at least one global M |1.0| are shown in Tables 1 and 2. A total of 109 were ultimately identified and categorized in terms of their cellular functions by using gene ontology and IPA annotation: The Venn-diagram for migration (45%), metabolism (45%), and cell death and survival (10%) are shown in Fig. 1. The relative abundances of the regulated genes between lung and MRGX-treated lung cancer cells determined by using a semi-quantitative analysis, as described previously [12], are listed in Table 1. Based on the criterion of a fold change > 1.0, 80 were up-regulated (Fig. 1A) whereas 29 were down-regulated in MRGX-treated lung cancer cells (Fig. 1B) compared to lung cancer.

3.2. Network analysis based on Gene Ontology analysis

To explore key MRGX-related proteins in the gene ontology analysis of gene functions, we used IPA to query 109 proteins belonging to the proteins up- and down-regulated by MRGX, resulting in a distinct interconnected network of 21 proteins (Fig. 2). There were quantitative and qualitative alterations in MRGX-treated lung cancer cells between the regulation groups. Among them, interleukin 8 (IL8) was identified as the center of the MRGX-related protein network in lung cancer cells. IL-8 is a lung giant-cell carcinoma-derived chemotactic protein that binds CXCR1 and CXCR2 [13]. In the present study, IL-8 is one of critical chemo-attractants responsible for leukocyte recruitment, cancer proliferation, and angiogenesis, and the potential mechanism of IL-8 production from human non-small-cell lung cancer was investigated [14]. IL-8 was decreased dramatically in the MRGX-treated lung cancer cells. The protein-protein network analysis suggests that IL-8 is a major protein that interacts with multiple proteins and that it is directly or indirectly down-regulated or up-regulated in MRGX-treat lung cancer cells. IL-8-linked proteins include chemokine (C-X-C motif) ligand 2 (CXCL2), MMP7 and PLAUR. PLAUR plays a key role in lung cancer [15]. However, the detailed mechanism has not been studied in lung cancer. Herein, the level of IL-8 in the MRGX-treat-

Table 1 Up-regulated gene list

Genbank Accession ID	Gene Symbol	Protein Name	Fold Change
NM_001159699	FHL1	four and a half LIM domains 1	2.158241
NM_001184717	TIPARP	TCDD-inducible poly (ADP-ribose) polymerase	1.982939
NM_000612	IGF2	insulin-like growth factor 2, somatomedin A	1.871624
NM_002203	ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	1.83122
NM_002526	NT5E	5'-nucleotidase, ecto (CD73)	1.808669
NM_032141	NSRP1	nuclear speckle splicing regulatory protein 1	1.734883
NM_000104	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	1.694663
NM_000104	UBASH3B	ubiquitin associated and SH3 domain containing B	1.555049
NM_003822	NR5A2	nuclear receptor subfamily 5, group A, member 2	1.503484
NM_000104	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	1.493132
NM_005891	ACAT2	acetyl-CoA acetyltransferase 2	1.490562
NM_004487	GOLGB1	golgin B1	1.477885
NM_005633	SOS1	son of sevenless homolog 1	1.463831
NM_004454	ETV5	ets variant 5	1.445291
NM_001723	DST	dystonin	1.432908
NM_001137550	LRRFIP1	leucine rich repeat (in FLII) interacting protein 1	1.410612
NM_004925	AQP3	aquaporin 3 (Gill blood group)	1.374904
NM_001173463	KIF21A	kinesin family member 21A	1.363929
NM_001105244	PTPRM	protein tyrosine phosphatase, receptor type, M	1.351404
NM_002483	CEACAM6	carcinoembryonic antigen-related cell adhesion molecule 6	1.34875
NM_022118	RBM26	RNA binding motif protein 26	1.333552
NM_003861	DCAF5	DDB1 and CUL4 associated factor 5	1.315943
NM_001023587	ABCC5	ATP-binding cassette, sub-family C (CFTR/MRP), member 5	1.313605
NM_003344	UBE2H	ubiquitin-conjugating enzyme E2H	1.287242
NM_001174087	NCOA3	nuclear receptor coactivator 3	1.286065
NM_001946	DUSP6	dual specificity phosphatase 6	1.253699
NM_003870	IQGAP1	IQ motif containing GTPase activating protein 1	1.250989
NM_001193455	NSUN2	NOP2/Sun RNA methyltransferase family, member 2	1.24729
NM_001025356	ANO6	anoctamin 6	1.2465
NM_021925	C2orf43	chromosome 2 open reading frame 43	1.242904
NM_001098272	HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)	1.210537
NM_005063	SCD	stearoyl-CoA desaturase (delta-9-desaturase)	1.201598
NM_014331	SLC7A11	solute carrier family 7 (anionic amino acid transporter light chain) member 11	1.186217
NM_001146276	NCEH1	neutral cholesterol ester hydrolase 1	1.185191
NM_000693	ALDH1A3	aldehyde dehydrogenase 1 family, member A3	1.183801
NM_004100	EYA4	eyes absent homolog 4	1.172664

NM_001032281	TFPI	tissue factor pathway inhibitor	1.171704
NM_001008938	CKAP5	cytoskeleton associated protein 5	1.171153
NM_001142568	BBX	bobby sox homolog	1.168988
NM_001113239	HIPK2	homeodomain interacting protein kinase 2	1.164367
NM_016284	CNOT1	CCR4-NOT transcription complex, subunit 1	1.162079
NM_004775	B4GALT6	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 6	1.160644
NM_014646	LPIN2	lipin 2	1.14518
NM_172037	RDH10	retinol dehydrogenase 10 (all-trans)	1.144987
NM_001005376	PLAUR	plasminogen activator, urokinase receptor	1.13818
NM_001077181	CDC14B	cell division cycle 14B	1.135517
NM_001083592	ROR1	receptor tyrosine kinase-like orphan receptor 1	1.135517
NM_007350	PHLDA1	pleckstrin homology-like domain, family A, member 1	1.131633
NM_020738	KIDINS220	kinase D-interacting substrate, 220kDa	1.11258
NM_017694	MFSD6	major facilitator superfamily domain containing 6	1.110798
NM_031897	CACNG6	calcium channel, voltage-dependent, gamma subunit 6	1.094036
NM_001130107	KLC1	kinesin light chain 1	1.093194
NM_001174159	SH2D4A	SH2 domain containing 4A	1.081308
NM_012090	MACF1	microtubule-actin crosslinking factor	1.078294
NM_001025081	MBP	myelin basic protein	1.077327
NM_001001894	TTC3	tetratricopeptide repeat domain 3	1.068548
NM_001190438	NCOR1	nuclear receptor corepressor 1	1.068334
NM_001077484	SLC38A1	solute carrier family 38, member 1	1.066673
NM_001962	EFNA5	ephrin-A5	1.064771
NM_014827	ZC3H11A	zinc finger CCCH-type containing 11A	1.058277
NM_015554	GLCE	glucuronic acid epimerase	1.051527
NM_012197	RABGAP1	RAB GTPase activating protein 1	1.04982
NM_001184998	KIAA0430	KIAA0430	1.049638
NM_015635	GAPVD1	GTPase activating protein and VPS9 domains 1	1.048175
NM_152641	ARID2	AT rich interactive domain 2	1.044772
NM_203365	RAPH1	Ras association (RalGDS/AF-6) and pleckstrin homology domains 1	1.041108
NM_145693	LPIN1	lipin 1	1.039824
NM_004462	FDFT1	farnesyl-diphosphate farnesyltransferase 1	1.03898
NM_024817	THSD4	thrombospondin, type I, domain containing 4	1.038791
NM_001002860	BTBD7	BTB (POZ) domain containing 7	1.038677
NM_001081550	THOC2	THO complex 2	1.035132
NM_001128626	SPIRE1	spire homolog 1	1.026552
NM_001197030	ANKHD1	ankyrin repeat and KH domain containing 1	1.023268
NM_001142614	EHBP1	EH domain binding protein 1	1.02271
NM_014689	DOCK10	dedicator of cytokinesis 10	1.013887

NM_004462	FDFT1	farnesyl-diphosphate farnesyltransferase 1	1.012972
NM_015491	PNISR	PNN-interacting serine/arginine-rich protein	1.012695
NM_004864	GDF15	growth differentiation factor 15	1.008396
NM_001031685	TP53BP2	tumor protein p53 binding protein, 2	1.004355
NM_016653	ZAK	sterile alpha motif and leucine zipper containing kinase AZK	1.001137

Table 2 Down-regulated gene list

Genbank Accession ID	Gene Symbol	Protein Name	Fold Change
NM_000585	IL15	interleukin 15	-1.00511
NM_006763	BTG2	BTG family, member 2	-1.01812
NM_005195	CEBPD	CCAAT/enhancer binding protein (C/EBP), delta	-1.02364
NM_001166599	FAM122B	family with sequence similarity 122B	-1.02554
NM_152603	ZNF567	zinc finger protein 567	-1.02634
NM_000366	TPM1	tropomyosin 1 (alpha)	-1.03063
NM_001416	EIF4A1	eukaryotic translation initiation factor 4A1	-1.03982
NM_001008405	BCAP29	B-cell receptor-associated protein 29	-1.05437
NM_001145275	ZFY	zinc finger protein, Y-linked	-1.06241
NM_152487	TMEM56	transmembrane protein 56	-1.0712
NM_002423	MMP7	matrix metalloproteinase 7 (matrilysin, uterine)	-1.07991
NM_000598	IGFBP3	insulin-like growth factor binding protein 3	-1.08553
NM_004617	TM4SF4	transmembrane 4 L six family member 4	-1.09854
NM_006761	YWHAE	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide	-1.11487
NM_014696	GPRIN2	G protein regulated inducer of neurite outgrowth 2	-1.13021
NM_004428	EFNA1	ephrin-A1	-1.18932
NM_020374	C12orf4	chromosome 12 open reading frame 4	-1.19108
NM_001143668	AMIGO2	adhesion molecule with Ig-like domain 2	-1.20343
NM_144643	SCLT1	sodium channel and clathrin linker 1	-1.2175
NM_003821	RIPK2	receptor-interacting serine-threonine kinase 2	-1.3159
NM_002522	NPTX1	neuronal pentraxin I	-1.36692
NM_006528	TFPI2	tissue factor pathway inhibitor 2	-1.45019
NM_005949	MT1F	metallothionein 1F	-1.45041
NM_001511	CXCL1	chemokine (C-X-C motif) ligand 1	-1.74981
NM_000584	IL8	interleukin 8	-1.94821
NM_002090	CXCL3	chemokine (C-X-C motif) ligand 3	-2.2981
NM_002089	CXCL2	chemokine (C-X-C motif) ligand 2	-2.38018
AF432419	C75F	C75F	-5.11758
AY227436	DSP1	drug-sensitive protein 1	-6.3106

AB014766

DERP12

dermal papilla derived protein 12

-6.74444

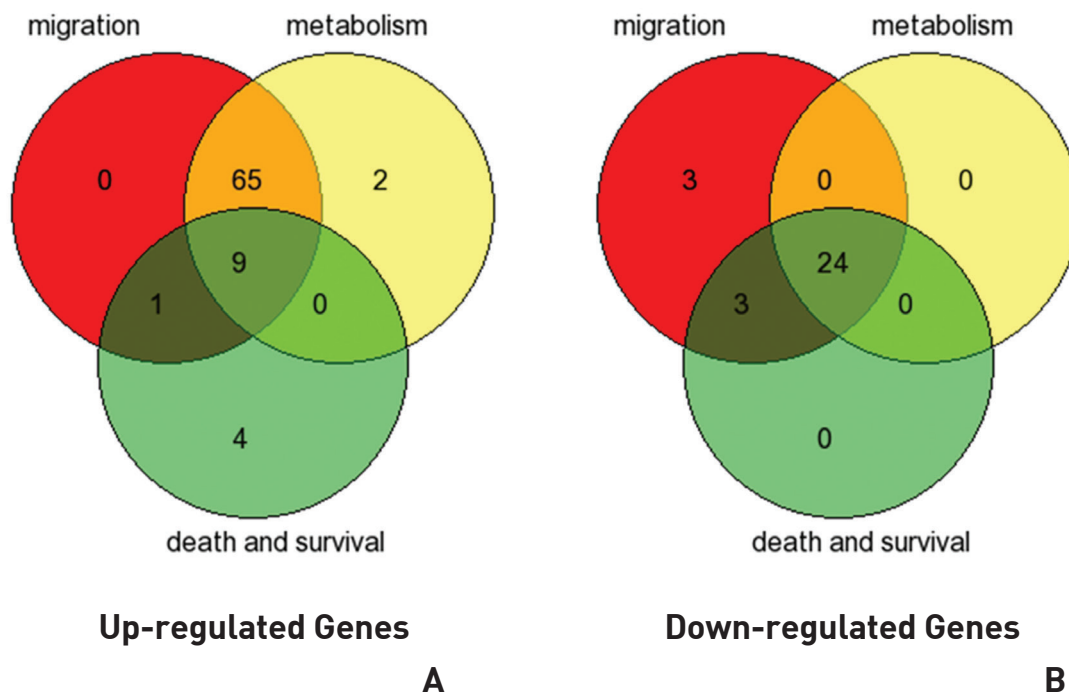


Figure 1 Venn diagram of a 2-fold changed gene based on cellular function using gene ontology. Venn diagrams show (A) genes up-regulated in MRGX-treated lung cancer cells by migration, metabolism, and cell death and survival in the microarray analysis, and (B) genes down-regulated in the microarray analysis.

ed lung-cancer cells was 1.9-fold down-regulated while PLAUR was up-regulated. The accumulation of PLAUR has been associated with lung cancer therapy.

4. Discussion

Microarray analyses may result in biased data, and inconsistent results are often obtained when different methods are used. Therefore, the data need to be validated with a specific method. Because we measured gene expression, we wanted to validate our screening results with conventional methods such as RT-PCR and Western blots. We selected genes from each of the two up- and down-regulated group of genes for validation by using semi-quantitative RT-PCR and Western blot analyses. Four genes were selected based on their high fold-change (a large number) in the analysis and the availability of commercial antibodies. IL-8, MMP7 and PLAUR are examples of up- and down-regulated genes. The mRNA levels of IL-8 and MMP7 were decreased in the MRGX-treated lung cancer cells, and the protein levels of IL-8 and MMP7 also decreased. The

semi-quantitative PCR and Western blot results showed that PLAUR was increased (Fig. 3).

5. Conclusion

In conclusion, most MRGX-responsive genes are up-regulated transiently in A549 cells, but are down-regulated in a sustained manner in lung cancer cells. These genes might be involved in the altered MRGX responsiveness observed during the cancer migration and metabolism processes. The roles of these genes in MRGX responses, including the cell cycle and cell growth, should be evaluated in further studies by modulating their expressions.

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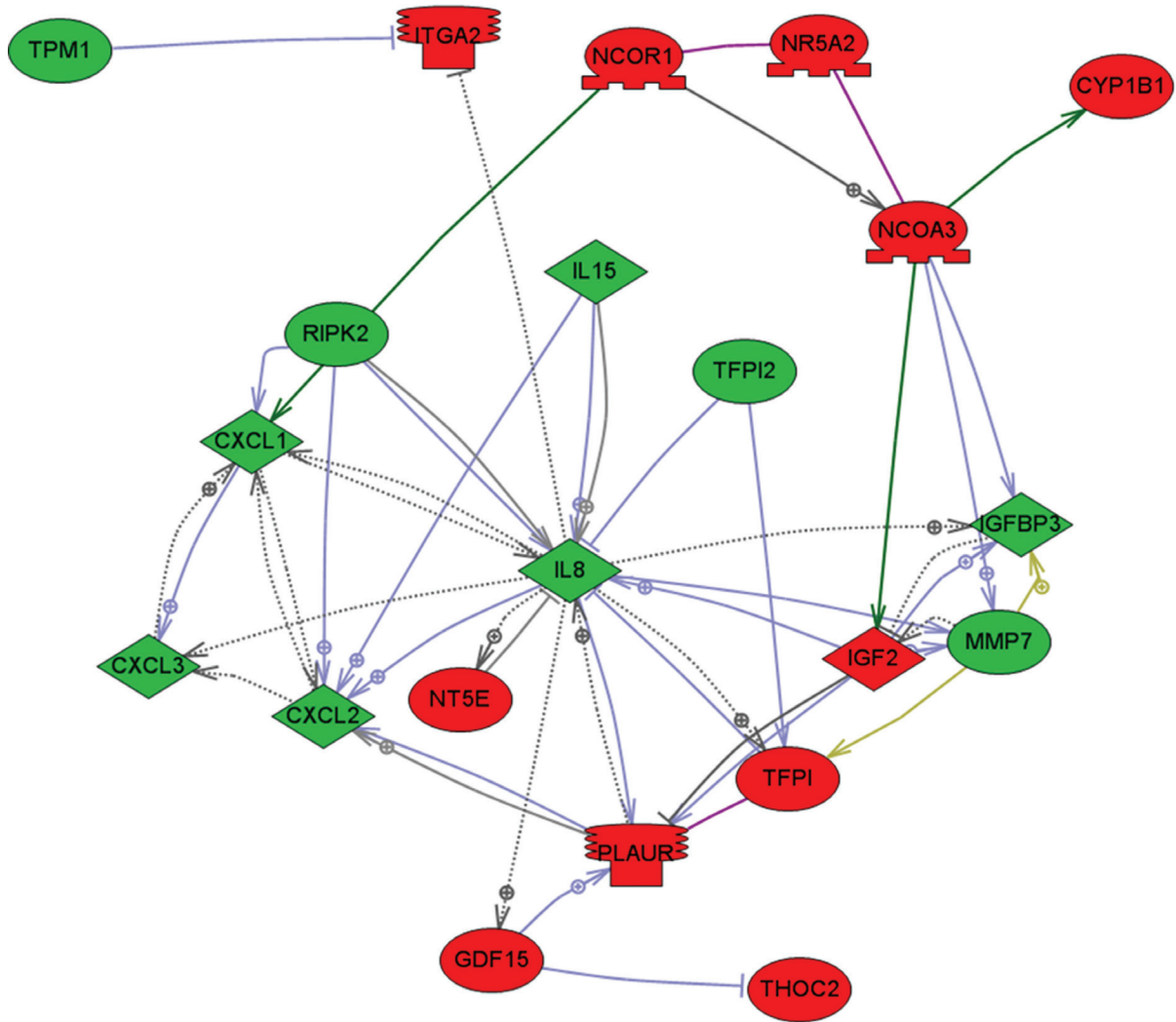


Figure 2 Network analysis based on a gene ontology analysis. Two-fold changed total proteins regulated by MRGX were queried by IPA, resulting in a distinct interconnected network of 21 proteins.

Protein		mRNA		
NT	MRGX	NT	MRGX	
				IL8
				MMP7
				PLAUR

Figure 3 Validation of microarray analysis results by using Western blot and RT-PCR analyses. Expressions of IL8 and MMP were examined by using a Western blot analysis with an anti-IL8 and MMP7 antibody (left panel) and by using a RT-PCR analysis (right panel). Expression of PLAUR was examined as described above. Beta-actin protein was used as a loading control for Western blot, and GAPDH was used as a normalization control for semi-quantitative PCR.

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