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# Microarray Analysis reveals that Dietary Retinoic Acid Suppresses Cancer-Related Gene Expression of the Lungs of Cigarette Smoke-Exposed Rats

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Previously we found that cigarette smoke depleted vitamin A in the lungs and induced tracheal precancerous lesions. To understand the molecular consequences underlying cigarette smoke-induced vitamin A depletion and its associated lung cancer risk, this study investigated the lung cancer-related genes in cigarette smoke-exposed rats with or without dietary retinoic acid, the active metabolite of vitamin A. Twenty-four male weanling rats were fed either a control or a retinoic acid supplemented diet. Half of each group was exposed to 40 commercial cigarettes/ d, 5 d/ wk. After 4 weeks, the rats were sacrificed and their lungs were immediately frozen. Total RNA was extracted and purified, from which cDNA was synthesized and labeled for gene expression analysis. Expressions of 120 genes were measured via a customized microarray. In lungs exposed to cigarette smoke, most of the genes involved in cell division, transcription and cell adhesion were up-regulated. The dietary retinoic acid treatment of the cigarette smoke-exposed lungs was found to down-regulate most of the genes involved in similar functions. In addition, retinoic acid down-regulated four genes, *Egr1*, *Fos*, *Icam1* and *Mmp9*, all of which were up-regulated by cigarette smoke. These findings suggest possible molecular mechanisms of cigarette smoke induced-lung cancer and define potential targets of retinoic acid anticarcinogenic actions.

**Keywords:** Cancer; Smoking; Retinoic acid; Cigarette; Genomics**Introduction**

Lung cancer is the leading cause of cancer-related death in both sexes worldwide [1], and in the United States [2]. Most cases of lung cancers are diagnosed after the disease has metastasized. Approaches that target the premalignant stage are needed to reduce the mortality of this disease. Extensive studies have indicated that vitamin A may be a potential agent in lung cancer prevention at an early stage. Vitamin A deficiency has been shown to be associated with bronchial metaplasia and an increased incidence of lung cancer [3]. From our previous study, we have found that exposure to cigarette smoke decreases vitamin A levels in the lungs and results in precancerous lesions in the trachea [4]. The molecular mechanisms by which lung cancer is induced by cigarette smoke and how that is related to vitamin A deficiency is complicated. Numerous components have been suggested to be involved in the process.

Microarray analysis is an effective method of analysis that allows investigators to simultaneously evaluate hundreds to thousands of gene expressions in a single experiment. This method helps identify new or known target genes, or gene function patterns along the lines of the pathways. This technique may prove helpful in determining the molecular mechanisms involved in lung cancer development. Customized microarrays were developed to narrow the focus of a full microarray to specific pathways of interest. In this study, we used customized microarrays to analyze one hundred twenty cancer related genes tailored to our specific research.

The purpose of this study was to identify candidate genes for lung cancer risk and determine whether cigarette smoke changed the expression of these genes. In addition, we determined whether dietary retinoic acid suppressed those changes. Categories of genes related to lung cancer risk were investigated, including: cell division; apoptosis; cell adhesion; tumor suppression. We also determined the gene expression of the retinoic acid receptors as well as growth and transcription factors.

**Methods and Materials****Animals and diets**

Male Sprague-Dawley weanling rats (Charles River Laboratories, Wilmington, MA) weighing about 50 g was housed individually in stainless steel cages at room temperature under a 12-hrs light: dark cycle (light 600 – 1800 hrs) and a relative humidity of 50%. Animal care and use were approved by the Institutional Animal Care and Use Committee of Kansas State University. Rats were fed a standard AIN-93G diet [5], with or without additional all-trans retinoic acid (10 mg/kg body weight, Sigma, St. Louis, MO). Food intake was recorded daily, and body weight was measured weekly. All rats were pair-fed so that there were no differences in food intake throughout the study.

**Cigarette smoke exposure conditions**

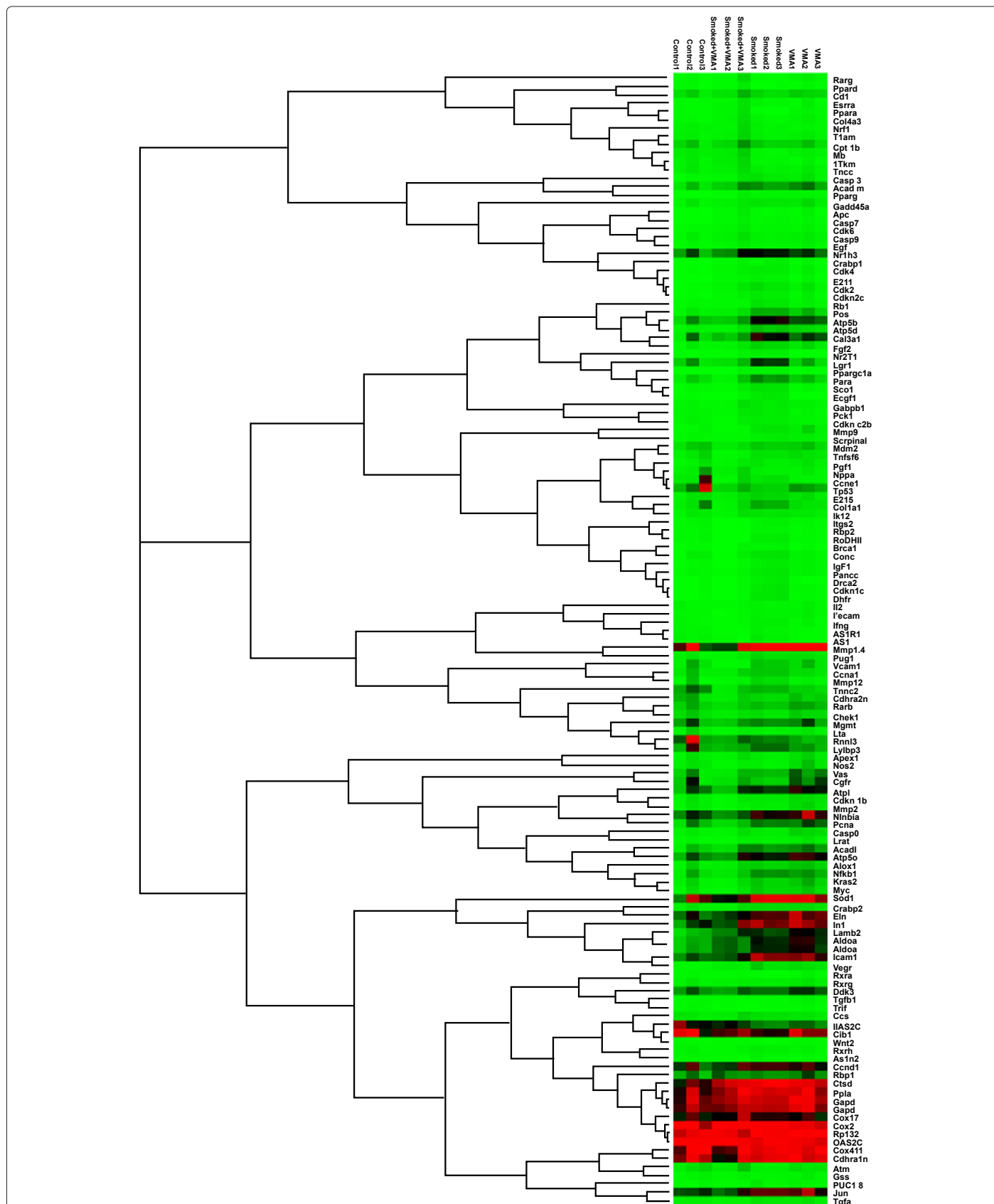
Total twenty-four rats were randomly assigned to four groups with six rats per group. Two of those treatment groups were exposed to smoke from two packs of cigarettes per day (nonfiltered commercial cigarettes, 40 cigarettes/ pack). The smoke exposure lasted for 4 weeks, 5d/wk. The cigarette smoke treatment has been previously described in detail [4]. Twelve rats were placed together in a plastic chamber measuring 65 cm long, 50 cm wide, and 45 cm high with three holes, two for holding the cigarette at one side of the chamber and another on the opposite

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**Figure 1:** Cluster analyses of the similarities of gene expressions in lungs among different groups. The column represents different treatment groups and the row represents different genes on each array. Red designates that genes were over expressed and green designates that genes were under expressed.

side of the chamber that was connected to a tube attached to a Leeson vacuum pump (model # A6C17EB20.1; Labconco, Kansas City, MO) for drawing the smoke. The rats were exposed to approximately 5 min of cigarette smoke (4 cigarettes), followed by 5 min of air, until all the cigarettes assigned to the group were consumed. One pack of cigarettes was considered one session and there was at least two hours of break between sessions. Rats in the control group were placed in another chamber, but were exposed to air only. The extent of exposure of rats to cigarette smoke was ascertained by measuring total particulate matter inhaled, and is reported in our previous study [4].

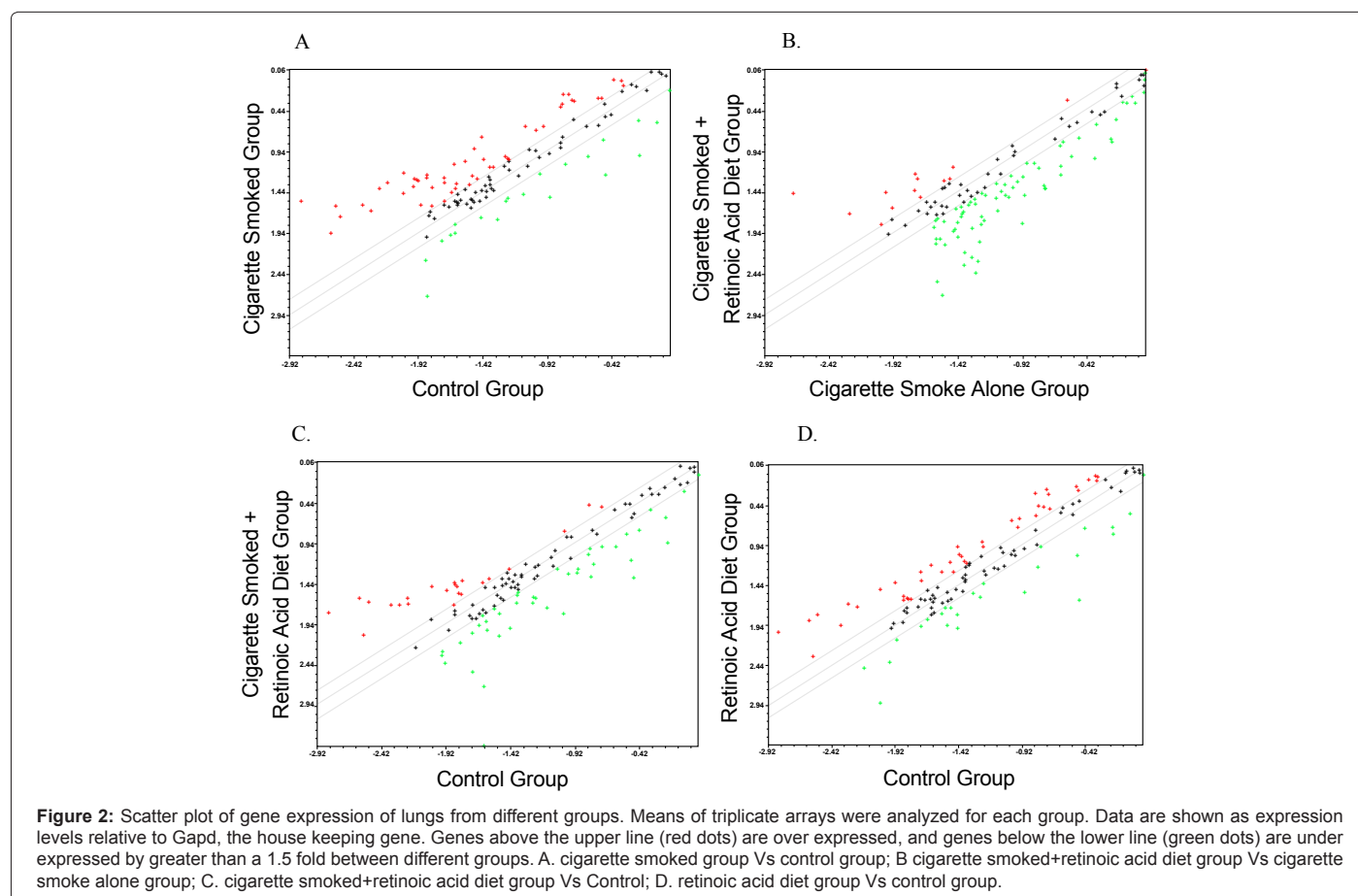
Cell cycle genes	Cell cycle checkpoints: Ccna1, Ccnc, Ccnd1, Ccne1, Cdk2, Cell cycle arrest: Cdkn1a, Cdkn1b, Cdkn2a, Ddit3, Gadd45a, Rb1
Regulation of cell cycle	Apc, Atm, Rb1, Brca1, Brca2, Ccnd1, Cdk4, E2f1, Fgf2, Gadd45b, Il1a, Il1b, Mdm2
Apoptosis genes	Atm, Bax, Bcl2, Myc, Tnf, Brca1, Casp3, Casp7, Casp8, Casp9, Ddit3, Mgmt
p53 Pathway	Bax, Cdkn1a(p21), E2f1, E2f5, Gadd45a, Igfbp3, Mdm2, Tnfrsf6, Tp53
NFkB Pathway	Icam1, Il2, TNFb, Myc, Nfkb1, Nfkbia, Nos2, Pecam, Tnf (TNFa), Vcam1
Proliferation indicators	Pcna, Fos, Jun
Growth factors	Fgf1, Fgf2, Egf, Egfr
DNA repair genes	Dhfr, Sod1, Gss
Retinoic acid receptors	Rara, Rarb, Rarg, Rxra, Rxrb, Rxrg, Rbp1, Rbp2, RoDHII, Crabp1, Crabp2, Lrat
Other	Kras, Cox2, c-myc, Vegf, EGF, EGFR, Egr1

Table 1: Selected lung cancer-related genes for microarray experiment.

### Probe synthesis and microarray hybridization

The right lobes of the lungs were immediately frozen in liquid nitrogen after harvesting. The day before the RNA extraction, the frozen lung lobe was transferred in solution containing RNAlater (Ambion, Austin, TX) at 4°C to prevent RNA degradation. The total RNA was extracted and purified from 150 mg of lung tissue through the use of an RNeasy Kit (Qiagen, Valencia, CA). The concentration and quality of RNA were checked by Nanodrop spectrophotometer and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The probe synthesis was done according to the manufacturer's protocol (SuperArray Bioscience, Frederick, MD). Briefly, 5 µg total RNA was used as template for biotinylated cDNA probe synthesis. RNA was reverse-transcribed by gene-specific primers with biotin-16-dUTP. Biotinylated cDNA probes were denatured and hybridized to customized gene-specific cDNA fragments spotted on the nylon membranes. The GEArray membranes were then washed and blocked with GEArray blocking solution, and incubated with alkaline phosphatase-conjugated streptavidin. The hybridized biotinylated probes were detected by chemiluminescent method using the alkaline phosphatase substrate, CDP-Star. The hybridized arrays were visualized using ChemiGlow West on a Fluorochem 8800 (Alpha Innotech Corporation, San Leandro, California) imaging system. The images of arrays are then saved as a grayscale file and ready for the intensity measurement by the software provided by SuperArray.

Each array membrane comprised 120 genes. In addition, five housekeeping genes were included to confirm the integrity of RNA and



correct loading of different samples. The selected lung cancer-related genes are listed in Table 1. Twelve customized cDNA GEArrays, three arrays for each group, were purchased from SuperArray.

### Microarray data analysis

Data analysis was performed on all 12 microarrays representing the lungs from different groups. As recommended by Superarray, the image data on each individual microarray was scaled to arbitrary target intensity, using the GEArray Suite software (version 1.1). The intensity of each array was measured by the software. The expression of Gapd, one of the house keeping genes as internal control designed by the company, was consistent for all the arrays showing less than 10% variation. The Gapd was then chosen for normalization. After all microarrays passing the quality acceptance criteria, normalization was performed using Gapd. The relative expression levels of different genes were estimated by comparing its signal intensity to that of Gapd. Clustering was used to compare the overall gene expression patterns among lungs from different groups.

### Statistical analysis

The normalized data were further transformed to log2 scale to achieve approximate normality. ANOVA and multiple comparisons were done in SAS PROC GLM to test pre-determined hypotheses. The p-values of the tests were imported into the software Q-value to compute q-values for each test of each gene [6]. The false discovery rate was specified to be 0.05 when calculating the q-values. The tests with q-values smaller than 0.05 were considered significant.

### Results

The similarities of gene expression profiles among different arrays were presented by clustergram (Figure 1). Gene expressions that are more similar are joined at lower heights in the dendrogram and those that are less similar are joined at higher heights. The pairs of genes that showed the most similarities and were joined with lowest height are Cdk2 and cdkn2c, Cdkn1c and Dhfr, Cdkn1b and Ddit2, Fanc and If1, Rbp2 and RoDHII, Tp53 and Ccne1, Fos and Fgf2, Kras2 and Myc.

A 1.5 fold change was chosen to determine the difference of gene expressions among different groups (Figure 2). When the cigarette smoke alone group was compared with control group, most of the genes were overexpressed, with 51 genes overexpressed and 20 under expressed (Figure 2A). When cigarette smoke exposure with retinoic acid diet group was compared with cigarette smoke alone group, majority of the genes were underexpressed. There were 71 underexpressed and 13 genes overexpressed (Figure 2B). When cigarette smoke with retinoic acid diet group compared with the control group, there were 22 genes over-expressed and 44 under-expressed (Figure 2C). The retinoic acid diet treatment alone resulted in 42 genes over-expressed and 24 under-expressed when compared with control group (Figure 2D).

The genes that had greater than 1.5 fold change between two groups were analyzed for statistical significance. All the genes that had significant differences between two groups ( $P < 0.05$ ) are shown in Table 2. When the cigarette smoke with retinoic acid diet group was compared with the cigarette smoke alone group, 10 genes were significantly down-regulated. For cigarette smoke alone group, 9 genes were significantly changed with 8 up-regulated and 1 down-regulated. Four genes that were up-regulated by cigarette smoke exposure were down-regulated by dietary retinoic acid: Fos, Icam1, Egr1, and Mmp9. When the cigarette smoke plus retinoic acid diet group was compared with control group, 2 genes were up-regulated (cdk4 and Casp7) and 1 was down-regulated (Tp53). The retinoic acid diet alone significantly up-regulated expressions of 3 genes: Casp7, Casp8, and Fos.

The functional classification of the genes that were statistically significant is listed in Table 3 corresponding to Table 2. The cigarette smoke group vs. the control group resulted in the significant up-regulation of 3 cell division genes, 3 transcription factor genes, 1 cell adhesion gene, 1 tumor apoptosis gene, and down-regulated one tumor suppressor gene. When the cigarette smoke with retinoic acid diet group was compared with the cigarette smoke alone group down-regulated genes included 2 cell division genes, 2 growth factor genes, 2 retinoic cell adhesion genes, 1 tumor suppressor inhibitor gene, 1 transcription factor gene, and 2 vitamin A metabolism genes. Cigarette smoke with dietary retinoic acid vs. the control group resulted in the significant

Symbol	Accession No.	Gene Identity	Functional Classification	Smoked vs Control	Smoked+Vita vs Smoked	Smoked+Vita vs Control	Vita vs Control
Tp53	NM_030989	Tumor protein p53	Tumor suppressor	↓	.	↓	.
Mdm2	XM_235169	Transformed mouse 3T3 cell double minute 2	Tumor suppressor inhibitor	.	↓	.	.
E2f1	D63165	E2F transcription factor 1	Transcription factor	↑	.	.	.
Fos	X06769	FBJ murine osteosarcoma viral oncogene homolog	Transcription factor	↑	↓	.	↑
Nfkb1	L26267	Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	Transcription factor	↑	.	.	.
Ecgf1	NM_001012122	Endothelial cell growth factor 1	Growth factor	.	↓	.	.
Igf1	NM_178866	Insulin-like growth factor 1	Growth factor	.	↓	.	.
Icam1	NM_012967	Intercellular adhesion molecule 1	Cell adhesion	↑	↓	.	.
Vcam1	NM_012889	Vascular cell adhesion molecule 1	Cell adhesion	.	↓	.	.
Egr1	NM_012551	Early growth response 1	Cell division	↑	↓	.	.
Cdk4	NM_031550	Cyclin-dependent kinase 4	Cell division	↑	.	↑	.
Mmp9	NM_031055	Matrix metalloproteinase 9	Cell division	↑	↓	.	.
Rbp2	NM_012640	Retinol binding protein 2, cellular	Vitamin A metabolism	.	↓	.	.
RoDHII	BC062000.	Retinol dehydrogenase type II	Vitamin A metabolism	.	↓	.	.
Casp7	NM_022260	Caspase 7	Apoptosis	↑	.	↑	↑
Casp8	NM_022277	Caspase 8	Apoptosis	.	.	.	↑

**Table 2:** Gene identity and function classification for the statistical significantly expressed genes in the lungs from different groups ( $P < 0.05$ ). Triplicate arrays were analyzed for each group, and data normalized based on Gapd within individual arrays. Dot represents no change between two compared groups. A ↑ indicates up-regulation and a ↓ indicates down-regulation.



Functional Classification	Smoked vs Control	Smoked+VitA vs Smoked	Smoked+VitA vs Control	VitA vs Control
Cell division	3(+)	2(-)	1(+)	.
Apoptosis	1(+)	.	1(+)	2 (+)
Growth factor	.	2(-)	.	.
Cell adhesion	1(+)	2(-)	.	.
Tumor suppressor inhibitor	.	1(-)	.	.
Tumor suppressor	1 (-)	.	1(-)	.
Transcription factor	3(+)	1(-)	.	1(+)
Vitamin A metabolism	.	2(-)	.	.

**Table 3:** Summary of functional classification for the statistically significantly expressed genes in the lungs corresponding to Table 1. Each number indicates the count of statistically significant genes. (+) indicates up-regulation while (-) indicates down-regulation.

up-regulation of 1 cell division gene, 1 apoptosis gene, and the down-regulation of 1 tumor suppressor gene. Retinoic acid diet alone vs. the control group found significant up-regulation of 2 apoptosis genes and 1 transcription factor gene.

## Discussion

In this study we investigated the regulation of genes related to cigarette smoke induced-lung cancer risk via customized microarrays and also what role dietary retinoic acid played in such an event in a rodent model. It was observed that cigarette smoke exposure for 4 wk substantially increased the expression of transcription factor genes and genes involved in cell division and apoptosis in the lungs. The dietary retinoic acid decreased the expression of genes involved in the similar functions.

This study provides the first evidence that the gene expression of p53 was down-regulated in cigarette smoke exposed-groups with or without dietary retinoic acid. The p53 gene, a well-known tumor suppressor gene, is especially important to lung carcinogenesis [7]. The loss and inactivation of p53 tumor suppressor functions is an essential contributor to transformation of normal human cells. Mutational alterations of p53 gene occur essentially in 100% of lung cancer as well [8], indicating that inactivation of p53 pathways occur as early events in the genesis of lung cancer [9]. The decreased gene expression of p53 in response to cigarette smoke suggests that the p53 gene may be a good target marker for early lung cancer detection.

The p53 gene itself is primarily targeted for mutational alterations in lung cancer, and indeed, p53 accumulation is believed to be a dominant step in retinoid-mediated cancer protection effects [10]. Amplification of the Mdm2 gene can accelerate p53 degradation and serve as alternate mechanisms for p53 functional inactivation [11]. In this experiment the gene expression of Mdm2 was decreased in the cigarette smoke with retinoic acid diet group when compared with cigarette smoke alone group suggesting that there was less degradation of p53 and therefore a predicted increase in p53. Future experiments measuring protein levels will be critical to ultimately determine how retinoic acid affects p53 in response to smoke exposure.

Another novel finding was that three transcription factors were up-regulated after four weeks of cigarette smoke exposure: E2F transcription factor 1 (E2f1), Fos, and Nuclear factor kappa b (Nfkb1). All three of these transcription factors are shown to be involved in the lung cancer development. E2f1 is a downstream factor of p53 signaling pathway involved in G1 progression of cell cycle. Overexpression of the transcription factor E2f1 correlated with increased tumor growth and aneuploidy via acting as a growth-promoting factor [12]. The Fos is one of the isomers of activator protein-1 (AP-1) that is frequently overexpressed in lung cancer cells [13]. The Nfkb1 gene is one of the critical genes that inhibit apoptosis to induce lung cancer [14]. Since

the exposure was only four weeks, the up-regulation of these genes may be early events in the development of lung cancer. Among these three transcription factors, only the gene expression of Fos was down-regulated in the cigarette smoke with retinoic acid diet group when compared with cigarette smoke alone group. Others have shown that all-trans retinoic acid down-regulates the transcriptional activation by AP-1 through the action of retinoic acid receptor beta [15]. Taken together, these genes may be potential targets for early detection of lung cancer risk and Fos may be a sensitive indicator of the effects of dietary retinoic acid.

Several sets of genes involved in tissue development/growth were also altered by cigarette smoke exposure and dietary retinoic acid. These include a couple of growth factors: Endothelial cell growth factor 1 (Ecgf1), and insulin growth factor 1 (Igf1). The Igf1 is recognized as a potent mitogen for lung cancer cells and there is good evidence that lung cancer cells produce Igf1 [16]. The Ecgf1 gene is a growth factor that promotes angiogenesis and the expression of Ecgf1 is important prognostic factors in lung cancer [17].

Another set of genes is two cell adhesion factors, vascular cell adhesion molecule 1 (Vcam1) and intracellular adhesion molecule 1 (Icam1). The occurrence of aberrations in cell adhesion is a critical phase in the invasion and metastasis of human cancer [18]. The gene expressions of Vcam1 and Icam1 were increased in cigarette smoke alone group but only the level of Icam1 was decreased when compared with cigarette smoke and dietary retinoic acid group. Therefore dietary retinoic acid may reduce lung cancer risk decreasing to some extent the gene expression of Icam1.

The last set of genes involved in tissue growth was cell division molecules, early growth response 1 (Egr1) and cyclin dependent kinase 4 (Cdk4). The increased gene expressions of these two genes in response to cigarette smoke exposure indicate an increase in cell division. Early growth response 1 belongs to a group of proteins that are involved in the progression through G1 phase of the cell cycle following growth factor stimulation. The dietary retinoic acid decreased the gene expression of Egr1 in lungs exposed to cigarette smoke, suggesting that Egr1 may be another target for retinoic acid action on lung cancer prevention. The gene expression of Cdk4 was increased in cigarette smoked groups and not affected by dietary retinoic acid. Previous research found that cdk4 forms a complex with the D-type cyclins involved in the control of cell proliferation during the G1 phase of cell division, and is believed to play an important role in lung cancer development [19].

It is interesting to note that the gene expression of matrix metalloproteinase 9 (Mmp9) was increased in response to cigarette smoke exposure and dietary retinoic acid decreased the gene expression. The increase of Mmp is found to be correlated with higher tumor grade and invasiveness [20]. Mmp2 levels are significantly elevated in the serum of patients with metastatic lung cancer, and in those patients

with high levels, response to chemotherapy is diminished [21]. These findings suggest that certain Mmps might result in an enhanced malignancy. Indeed, Mmp9 which in this study was shown to be up-regulated by cigarette smoke and down-regulated by retinoic acid has already been found to be associated with malignant lung cancer tissues [22].

There were decreases of two retinoic acid metabolism genes: retinol binding protein 2 (Rbp2) and retinol dehydrogenase type II (RoDHII) in response to dietary retinoic acid with cigarette smoke exposure compared to smoking. Retinol binding protein 2 binds with retinol and is responsible for the transport of vitamin A within the cell. Retinol dehydrogenase II catalyzes the conversion of retinol to retinal, the first step in retinoic acid synthesis. The decreased expression of both genes suggests that the retinoic acid supplementation decreased the need for transport of retinol to the lung and decreased conversion to retinoic acid.

In summary, the findings from this study suggest several target genes that can be investigated in the future lowering of lung cancer risk induced by cigarette smoke. There was an increase of gene expression of Egr1, Fos, Icam1 and Mmp9 in response to cigarette smoke exposure and decrease with additional dietary retinoic acid, indicating that they may be targets of retinoic acid anticarcinogenic actions. The current study will help define research avenues in the pursuit of the molecular mechanism of lung cancer development.

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