Systems Toxicology Approaches Enable Mechanistic Comparison of Cigarette Smoke-induced and Spontaneous Lung Tumors in the A/J Mouse Model

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Introduction

The A/J mouse strain is frequently utilized as an animal model for cigarette smoke (CS)-associated lung tumorigenesis. However, the high susceptibility of the strain to develop spontaneous lung tumors creates a challenge in understanding the mechanisms underlying lung tumor formation following CS exposure. This study demonstrates how systems toxicology approaches not only enable a better mechanistic understanding of CS-related lung tumorigenesis in this animal model, but also to distinguish between CS-related and spontaneous tumors. Male A/J mice were exposed whole-body mainstream CS. An increased tumor incidence and multiplicity was observed in mice exposed to CS compared to an air exposed sham group. Transcriptomic analysis on 30 pairs of lung lesions (nodular hyperplasia, adenoma, and carcinoma combined) and surrounding, non-tumorous parenchyma samples for both exposed and sham animals identified 269 genes to be significantly differently expressed between the two groups (FDR<0.01). Biological networks related to a suppression of the humoral immune response and a perturbation of both sphingolipid and glycosaminoglycan metabolism were associated with CS-related tumors. A 50-gene signature was generated and shown to be able to discriminate between the CS-related and spontaneous lung tumors. Furthermore, 70 miRNAs were identified that behaved differently in the tumor as compared to parenchyma tissues in response to CS exposure.

Materials and Methods

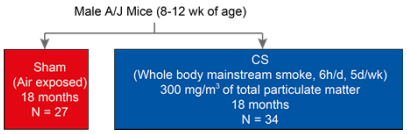

Figure 1. Experimental groups and procedure

Animal Experiments
The experimental groups and procedure are shown in Figure 1. The design of the study has been described previously[1, 2]. The reference 3R4F cigarettes (University of Kentucky, Lexington, KY, USA) were used in the study. The study was approved under the Belgium Law on Animal Protection and performed in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Histopathology
Histopathological evaluation was performed by a trained and certified pathologist in a blinded manner as described previously[1, 2]. Two animals in the sham group were excluded from the analysis due to insufficient instillation of the lung and the presence of a primary non-lung tumor. Pulmonary proliferative lesions (PPLs) were classified following the International Classification of Rodent Tumors[3].

mRNA and miRNA Analysis
The mRNA analysis has been described previously[1, 2]. For miRNA analysis, the dual-channel Exiqon miRCURY LNA™ platform was chosen for hybridization, which was performed directly by Exiqon (Vedbaek, Denmark).

Statistical and Computational Analysis

Tumor Incidence and Multiplicity
Fisher Exact Test for overall analysis followed by pairwise comparison was applied to calculate tumor incidence (the number of animals having at least 1 lesion divided by the group size). ANOVA followed by pairwise comparison using Tukey test was applied to calculate tumor multiplicity (the number of lesions per individual animal summarized per exposure group and divided by the group size). These analyses have described previously[1, 2]

Gene Expression Analysis
Microarray expression values were generated from the CEL files using background correction, quantile normalization, and median polish summarization. Interaction effects between tissue type (tumor (T) or parenchyma (P)) and exposure (sham or CS) on gene expression were determined using a linear model:

$$GxP = \beta_0 + \beta_1 \times CeT + \beta_2 \times doL + \beta_3 \times doM + \beta_4 \times doH + \beta_5 \times CeT:doL + \beta_6 \times CeT:doM + \beta_7 \times CeT:doH + \varepsilon$$

where L, M, and H denote low, medium and high concentrations, respectively, and ε is the error term. The term *CeT* represents the tumor effect, and the terms *doL*, *doM* and *doH* are the CS dose effects. The interaction coefficient *CeT:doH* [(high dose in tumor – sham in tumor) - (high dose in parenchyma – sham in parenchyma)] describes the difference in gene expression between tumor and parenchyma for the mice exposed to a high dose of CS compared to sham. The cutoff for the false-discovery rate (FDR) was set at 0.01. Ingenuity Pathway Analysis Analysis (IPA®) was utilized to interpret the biological functions of the genes that were significantly altered under the interaction analysis.

Gene Signature Generation
A gene signature to discriminate between spontaneously and CS- exposed tumors was extracted from the interaction coefficient *CeT:doH* as follows: First, the FDR cutoff was set at 0.2 to filter genes. Then, a gene signature consisting of 50 genes was generated by using an in-house developed program, GenSigPred, in which supervised machine-learning approaches including SAM[4] and support vector machine[5] were applied in a 10-fold validation procedure.

miRNA Expression Analysis
The same linear model used for gene expression analysis was applied for the analysis of miRNA. A less stringent cutoff for FDR was set at 0.05.

References

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Results

Group	Lung tumor incidence		
	Nodular hyperplasia	Bronchiolo-alveolar adenoma	Bronchiolo-alveolar carcinoma
Sham	7/25	13/25	10/25
CS	11/34	33/34*	20/34

Table 1. Lung tumor incidence. The incidence of bronchiolo-alveolar adenomas in CS-exposed mice is statistically significantly higher than that in sham (*p<0.001).

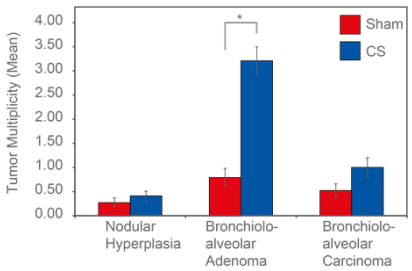


Figure 2. Lung tumor multiplicity in A/J mice exposed to air (red bars) or to CS from 3R4F cigarettes (blue bars). The multiplicity of bronchiolo-alveolar adenomas in cigarette smoke-exposed mice is statistically significantly higher than that in mice exposed to air (*p<0.001).

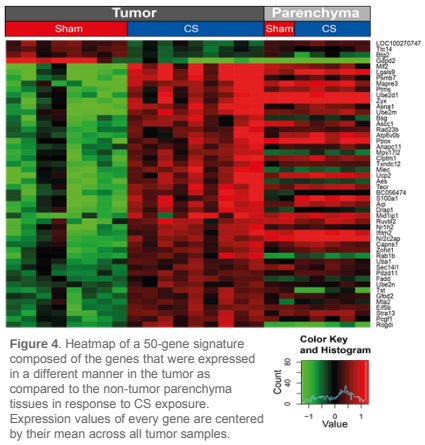


Figure 4. Heatmap of a 50-gene signature composed of the genes that were expressed in a different manner in the tumor as compared to the non-tumor parenchyma tissues in response to CS exposure. Expression values of every gene are centered by their mean across all tumor samples.

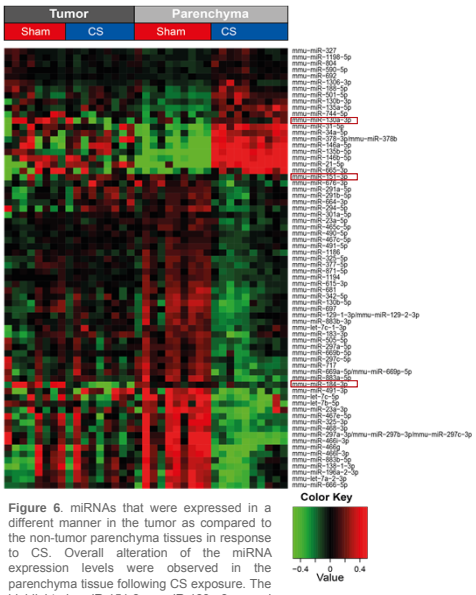
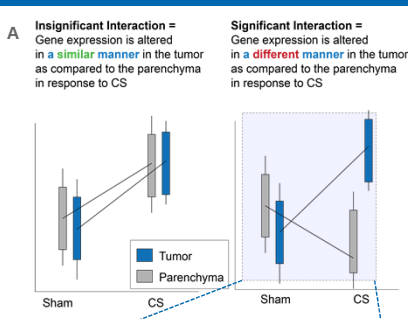


Figure 6. miRNAs that were expressed in a different manner in the tumor as compared to the non-tumor parenchyma tissues in response to CS. Overall alteration of the miRNA expression levels were observed in the parenchyma tissue following CS exposure. The highlighted miR-151-3p, miR-130a-3p, and miR-184-3p have been previously implicated in carcinogenesis and are shown in the network (Figure 5).



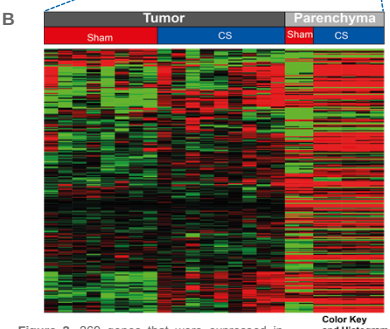


Figure 3. 269 genes that were expressed in different manner in the tumor as compared to the non-tumor parenchyma tissue in response to CS exposure (with significant interaction). **A)** Illustration of the interaction effects between tissue types and exposures. **B)** Heatmap of genes with significant interaction that were used for mechanistic investigation (Figure 5).

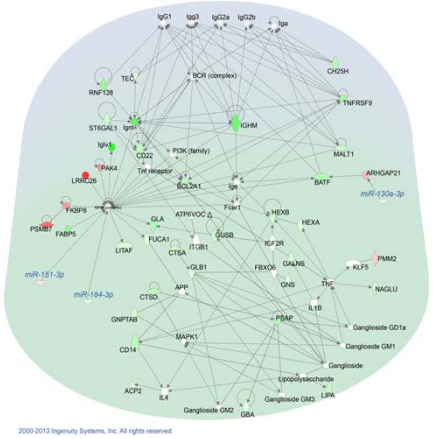


Figure 5. A network highlighting suppression of immune response and decreased glycosphingolipid metabolism from Ingenuity Pathway Analysis (IPA®).
Molecules in the network represent the differentially expressed genes in CS-induced tumors when compared to their matched non-tumor parenchyma tissues after subtracting the changes in the sham animals. Green and red indicate significantly decreased and increased fold-change, respectively, of the gene expression affected by CS that behaves differently in the tumors vs. parenchyma. Color intensity qualitatively represents the degree of fold-change. Straight and dashed lines specify direct and indirect interaction, respectively.

Conclusion

This study shows that systems toxicology approaches not only serve as valuable tools to evaluate the differing mechanisms underlying lung tumorigenesis between the development of spontaneous lung tumors and CS-related lung tumorigenesis. Furthermore, the systems approach also provides a means to distinguish between these two tumor types. IPA® indicated an overall suppression of the cell-mediated and humoral immune response which was accompanied by a disruption of sphingolipid and glycosaminoglycan metabolism in tumors of CS-exposed A/J mice. Given the different characteristics of these tumors and the utility of the gene signature, we propose that the A/J mouse could be a useful and sensitive tool for assessing reduced disease-risk associated with novel Modified Risk Tobacco Products.

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