Quantitative Proteomics To Investigate The Effects of a Prototype Modified Risk Tobacco Product (pMRTP) And Smoking Cessation On Mouse Lung Proteome.

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Introduction and Objectives

Chronic obstructive pulmonary disease (COPD) is defined by the World Health Organisation (WHO) as a lung disease characterized by chronic obstruction of lung airflow that interferes with normal breathing and is associated with narrowing of the small airways, chronic bronchitis, and the development of alveolar emphysema. Cigarette smoke is the primary risk factor in the development and progression of COPD. PMI is developing potentially modified risk tobacco products (MRTPs) in an effort to reduce the risk of smoking-related diseases in smokers who switch from combustible cigarettes to the MRTP. In this study, the impact on the development of emphysema/COPD following inhalation of smoke from a reference cigarette (3R4F, University of Kentucky) and aerosol from prototypic modified risk tobacco product (pMRTP), was evaluated in C57Bl/6 mice over a period of 7 months. After 2 months of exposure to 3R4F smoke, switching and cessation groups were exposed to pMRTP aerosol or filtered air (sham), respectively, iTRAQ and 2D-PAGE methods were used for relative quantification of differentially expressed proteins in lung of C57BL/6 mice and RPPA was used for verification.

The main objectives of the study were:

- To identify differentially expressed proteins in the different experimental groups using iTRAQ and 2D PAGE and compare both techniques.
- To verify and confirm the identified differential expressed proteins using reverse phase protein array (RPPA).
- To determine the effects of pMRTP and switching to pMRTP in comparison with cigarette smoke (CS) on the lung proteome.

Methods

The mice were exposed to smoke from 3R4F (750 micrograms/liter of total particulate matter – TPM), aerosol from pMRTP or filtered air for 4 hours per day, 5 days per week, up to 7 months*. Both tobacco products used had the same nicotine concentration in smoke and aerosol - 34.4 micrograms/liter. After 2 months of exposure to 3R4F smoke, switching and cessation groups were exposed to pMRTP aerosol or filtered air, respectively. Right lung samples from month 1, 3, 5 and 7 were analyzed*.

* All procedures involving animals were performed in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) accredited, AVA-licensed (Agri-Food & Veterinary Authority of Singapore) facility with approval from an Institutional Animal Care and Use Committee, in compliance with the National Advisory Committee for Laboratory Animal Research Guidelines on the Care and Use of Animals for Scientific Purposes..

iTRAQ: 50 µg of extracted proteins from the 5 respective treatment groups were reduced, alkylated and trypsin digested in parallel followed by labelling using iTRAQ tags and samples were pooled. Finally, the tagged peptides pool was analyzed by LC MS/MS to be identified and quantified.

2D-PAGE: 150 µg of protein was loaded and separated on 11 cm strip, 3-10 NL, then on 13 cm 12% SDS-PAGE and finally stained with Sypro Ruby. SameSpots software (TotalLab) was used for the detection of differentially expressed proteins by comparison to the control sample (Sham). Tryptic digested samples were analyzed using MALDI TOF/TOF-MS and proteins were identified using Mascot search engine against the Uniprot Mouse database.

RPPA: Spotting of normalized lung lysates in serial dilution on Chip using the GeSim Nanospotter. After Blocking, the primary Abs were applied followed by the 2nd fluorescent labelled Abs. The signal was detected and the images were analyzed using the Zeptoreader .

Figure 1. Treatment groups and experimental design and analyzed time points.

Results

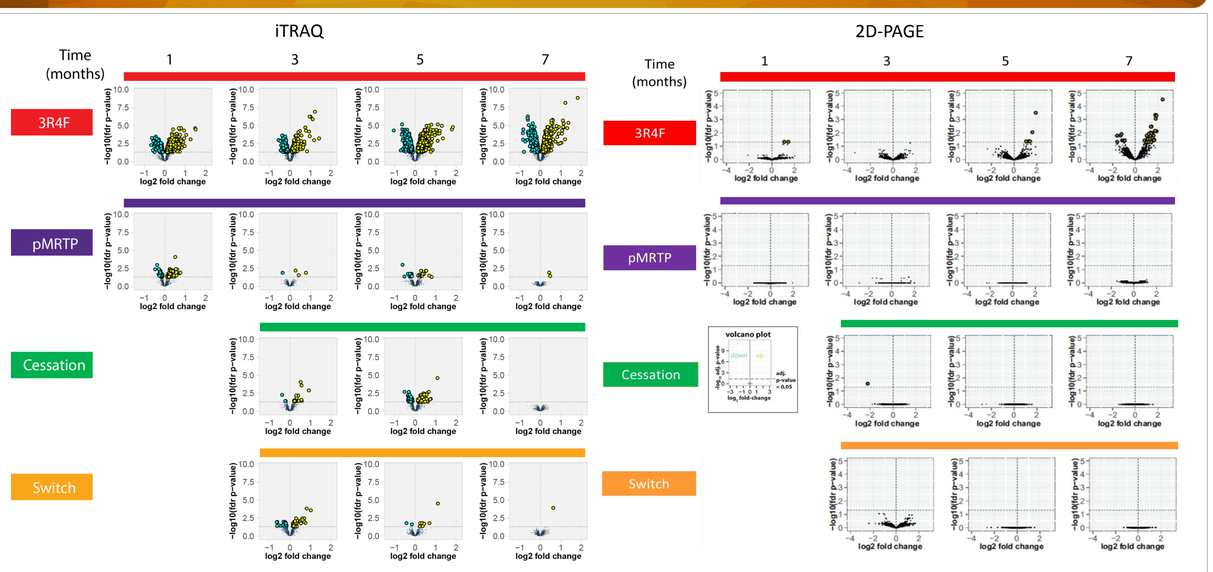

Figure 2. Volcano plots showing the effect amplitude (log2 fold-change, x-axis) and significance (–log10 adjusted p-value, y-axis) in the exposure groups compared with sham exposure. The significance threshold of the fdr-adjusted p-value = 0.05 (1.3 in log10 space) is indicated and significantly up and downregulated proteins (protein spots) detected by iTRAQ and 2D PAGE are shown as yellow and blue dots, respectively.

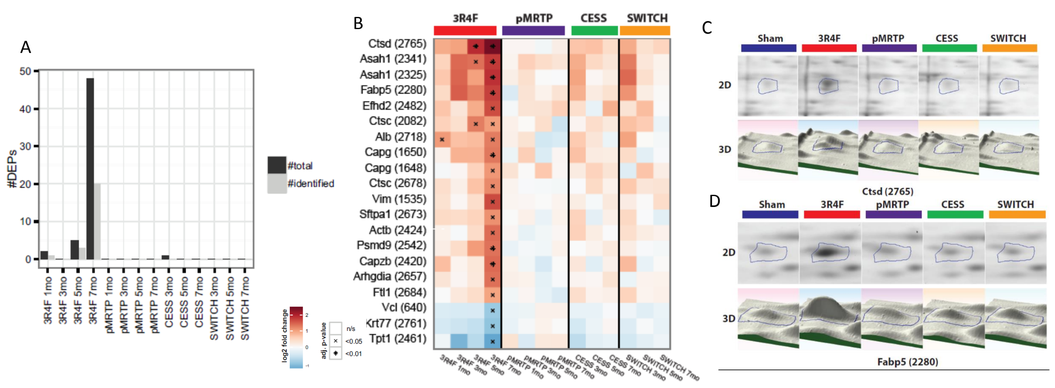

*Figure 3: 2D-PAGE captured the lung proteome response to CS and pMRTP exposure. (A) Summary of the number of detected differentially expressed proteins (DEPs) in total and that could be identified by MALDI-TOF. (B) Protein response heatmap for all identified proteins with significant differential expression by 2D-PAGE (fdr-adjusted p-value < 0.05). Each row represents a protein spot (labeled with protein name and spot number), each column represents an exposure group vs. sham comparison. The log2 fold-change is color-coded (see color key) and the statistical significance indicated (× = adj. p < 0.05; * = adj. p < 0.01). Note that for the basic statistics we counted different protein spots for the same proteins separately. (C) Representative spot intensities of protein Ctsd (spot #2765) for one replicate of the 7 month time point (2D- and 3D- representation). (D) As C, but for Fabp5 (spot #2280).*

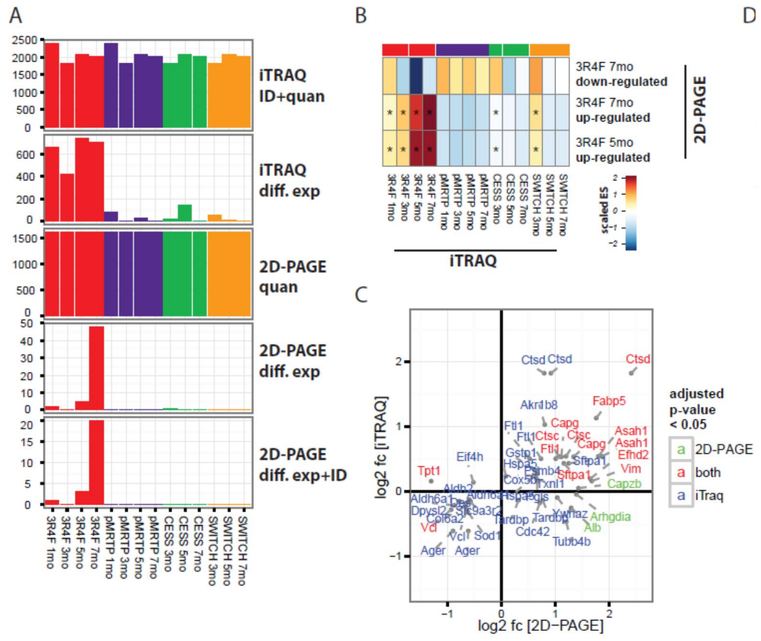

Figure 4. Comparison of proteome response profiles measured by 2D-PAGE and iTRAQ. (A) Comparison of the number of identified, quantified, and differentially expressed proteins measured by 2D-PAGE and iTRAQ. From top to bottom, the bar chart shows for each exposure group vs. sham comparison: the number of identified and reliably quantified proteins from iTRAQ , the number of differentially expressed proteins identified by iTRAQ (fdr-adj. p-value < 0.05), the number of quantified protein spots from 2D-PAGE, the number of differentially expressed protein spots from 2D-PAGE, and the number of differentially expressed protein spots to which a protein identifier could be assigned. (B) Comparison of 2D-PAGE with iTRAQ differential expression results. Protein sets are defined based on the differentially expressed proteins from the 2D-PAGE analysis (3R4F 5 months upregulated, 3R4F 7 months up/downregulated). Gene/protein set analysis (GSA) of these sets was performed for each comparison of the iTRAQ data and the 551 mean fold-change, scaled per gene set, was color coded (ES, enrichment score) and the statistical significance indicated (= fdr-adjusted p-value < 0.05). (C) Scatter plot comparing the fold-change responses of differentially expressed proteins from the 3R4F 7 months vs. sham comparison from 2D-PAGE and iTRAQ data. The color of the protein label indicates whether differential expression was identified by 2D-PAGE (green), iTRAQ (blue), or both (red).*

Results

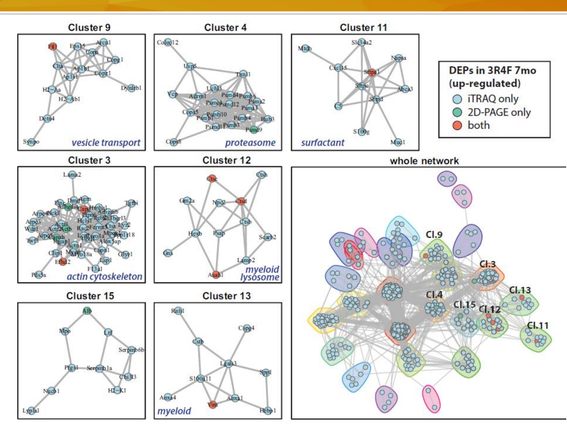

Figure 5. Functional network embedding for interpretation of the detected exposure response (3R4F 7 months vs. sham). Functional association clusters were identified for the significantly upregulated proteins in the 2D PAGE and iTRAQ data (3R4F 7 months vs. sham) . Functional protein clusters are color-coded and clusters with differentially expressed proteins identified by 2D-PAGE are labeled.

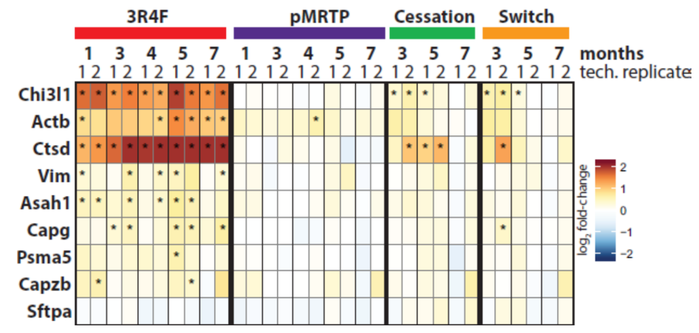

Figure 6. Reverse-phase protein array (RPPA) results for selected core cluster components. RPPA result heatmap with target proteins in rows and exposure comparisons in columns. Results of two technical replicates are included and labeled correspondingly. The fold-change in each exposure group compared with the sham group at the same time point is color-coded (see color key) and statistically significant differences are marked (= fdr-adj. p-value < 0.05). Statistics based on t-test of log-transformed data; only comparisons with at least three not missing values per group are considered.*

Discussion

iTRAQ workflow resulted in the identification and quantification of more proteins compared to 2D-PAGE.
The functional network analysis of cigarette smoke (CS) exposure detected by both proteomics methods shows that the response overlapped.
The utilization of both the 2D-PAGE and iTRAQ methods led to confirmation and extension of some impacted biological processes such as immune (myeloid)-, surfactant-, proteasome-, and actin cytoskeleton-related processes.
The effect on the proteasome-, and actin cytoskeleton-related processes was confirmed by RPPA approach.

Conclusions

iTRAQ workflow clearly provided greater coverage of the CS-induced effects. Nevertheless, the 2D-PAGE/MALDI MS/MS results proved useful to support the overall trends, especially the lower effect of pMRTP aerosol than CS on the lung proteome is observed, and the CS effect on specific functional categories such as actin cytoskeleton regulation.
RPPA proved to be an relevant method for verifying and confirming identified selected proteins from the MS approaches.
While the field of toxicoproteomics is employing preferentially gel-free methods (labeled and label-free), robust 2D-PAGE retains a role as a complementary method for intact proteins.
All methods show a less effect on lung proteome of exposure to pMRTP aerosol than to CS when compared to Sham.

Reference

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