In Vitro Model for Identifying Subchronic Carbon Nanotube Exposure and Lung Cancer Gene Markers

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ABSTRACT

Exposure to numerous engineered nanomaterials (ENMs) results in human health concerns (ie. lung fibrosis and cancer); however, comparative *in vivo* carcinogenesis studies are resource consuming. An integrative subchronic *in vitro* exposure model, coupled with toxicogenomic and correlation feature selection strategies was developed to identify particle-specific, key gene markers for carbon nanotube (CNT) induced carcinogenic potential. Single and multi-walled CNT (SWCNT and MWCNT, respectively), asbestos (ASB), ultrafine carbon black (UFCB) treated and control SAEC genome expression signatures were subjected to comparative marker analyses to identify genes with highly correlated expression for each treatment. Specific markers and genome profiles were subjected to Ingenuity Pathway Analysis to assess marker performance. Key marker gene subsets and disease marker expression were successfully validated. Toxicogenomic signature profiling in a subchronic *in vitro* exposure model can potentially aid in assessing detection of early CNT disease in workers during nanomaterial manufacturing.

Disclaimer: The findings and conclusions in this abstract are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

Keywords: carbon nanotube, asbestos, in vitro model, biomarkers, toxicogenomics

1 INTRODUCTION

Concern for increased risk of carbon nanotube (CNT)-associated lung disease, including carcinogenesis, has been raised due to asbestos-like high aspect ratio, deep pulmonary deposition, biopersistence, and *in vivo* MWCNT tumor promotion [1,2]. Numerous *in vivo* studies report that MWCNT injections at extremely high doses result in increased risk of mesothelial hyperplasia, granulomas and tumor development [3,4]. CNT aspiration and inhalation exposure causes transient inflammation, interstitial fibrosis and movement of CNT fibers out of the pleural cavity [5,6]. In the presence of a tumor initiator, MWCNT were found to promote bronchial/alveolar carcinomas [2]. Our previous studies found that sub-chronic *in vitro* exposure to dispersed single- (SWCNT) and multi-walled (MWCNT) CNT resulted in neoplastic-like and malignant transformation in human small airway (SAEC) and bronchial epithelial cells [7,8]. Very few *in vivo* studies have identified and compared 'omic' signature response of CNT vs. asbestos vs. other carbon nanomaterials [9,10].

As development of novel ENM commercial, industrial and biomedical applications become mainstream, identification and assessment of potential adverse effects, including cancer, upon ENM exposure becomes paramount in protecting ENM worker health. Thus, rapid high-throughput screening methods to 1) identify those ENMs with unique intermediate to long-term health effects and 2) characterize both exposure and disease markers associated with ENM exposure is urgently needed.

Here, we use a sub-chronic *in vitro* exposure model, coupled with toxicogenomic profiling and correlation feature selection strategies to identify particle-specific, key gene markers from SWCNT-, MWCNT-, crocidolite asbestos (ASB)-, ultrafine carbon black (UFCB)-treated and control SAEC genome expression signatures. By comparing these *in vitro* particle specific biomarkers to *in vivo* expression data via an 'omics' approach, *in vitro* sub-chronic exposure models show promise in assisting development of ENM occupational and environmental risk assessments [11,12].

2 METHODS

2.1 Cell Culture and Sub-Chronic Exposure

Immortalized human small airway epithelial cells (SAECs) were cultured in SABM medium supplemented with growth factors (Lonza) and 1% penicillin/ streptomycin. Cells were cultured in a humid, 5% CO_2 atmosphere at 37 ºC throughout the study.

SWCNT (HiPCO; CNI) possessed dispersed mean particle width of 270 nm, 1.08 µm length, and 440-1020 m^2/g surface area (SA). MWCNT (Mitsui #7) possessed dispered 78 nm width, 5.1 µm length and 26 m^2/g SA. UFCB (Elftex 12; Cabot) displayed dispersed 700 nm width and 43 m^2/g SA.. ASB was acquired from NIEHS and had 210 nm width, mean 10 µm length and 9.8 m^2/g SA.

SAECs ($5x10^4$ in triplicate) were sub-chronically exposed to four different particle treatments plus two control treatments continously for 6 m [8]. Briefly, each particle was suspended in filtered, sterile water to acquire a 0.1 mg/mL stock solution. In addition, each stock contained a final concentration of 150 µg/mL Survanta®, a natural

lung surfactant, to adequately disperse nano-sized particles [7,8]. To expose cells, each stock was sonicated for 5-10 seconds using a microtip horn then diluted in warm cell culture medium to 0.02 μg/cm^2 in 2 mL medium/well in 6-well plates. Saline-only (SAL) and dispersant-only (DISP) exposed cells served as passage controls. Cells were re-exposed to each dispersed particle treatment every 3 d and passaged every 6-7 d.

2.2 Cancer Hallmark Phenotype Assessment

To assess ENM-induced neoplastic transformation, SAECs from each 6 m treatment were phenotypically screened for cancer hallwarks [7,8]. WST-1 and trypan exclusion assays measured proliferation, transwell inserts with and without Matrigel assessed invasion and migration ability, while soft agar colony formation assay assessed attachment-independent growth ability. Morphological transformation was assessed using pre-validated methods to determine colony formation unit frequency and transformation frequency. One-way analysis of variance determined differences among means followed with appropriate post-hoc Dunnett's or Tukey-Kramer HSD (α = 0.05). Analyses were performed in SAS JMP (ver. 10).

2.3 Whole Genome Signature Analysis

RNA from 6 m exposed cells was collected and isolated with TriZol reagent, then shipped frozen to ArrayStar, Inc. (Rockville, MD). Cy3 labelled cDNA were hybridized to NimbleGen Human Whole Genome 12x135k Arrays. Raw intensity expression data were acquired by an Axon GenePix (Molecular Devices), evaluated and normalized in NimbleScan. Differentially expressed genes (DEGs) exhibited a ≥ ±2-fold change and passed a t-test compared to DISP control ($p \leq 0.05$). Gene ontology and gene signaling network (GSN) analysis were conducted in Ingenuity Pathway Analysis (IPA) [8].

2.4 Comparative Marker and Class Neighbor Analyses

Since our evalution of both cancer hallmark phenotypes and GSN analysis indicated a more aggressive tumor-like behavior in CNT-exposed cells with different signaling mechanims than ASB-exposed cells, we employed two separate correlation feature selection analyses to identify genes that displayed particle-specific gene expression signature. Normalized expression values were uploaded into GenePattern (Broad Institute, Cambridge, MA), then analyzed using comparative marker and class neighbor analyses [13]. Genes were scored and ranked using correlation, t-test and p-value procedures, then corrected using FDR. Top-ranked gene functions were annotated using both GeneSpring and IPA.

2.5 Knowledge-based Biomarker Network Analysis and Validation

After multistep cross validation, specific treatment markers (≤ 100 genes) and genome profiles were subjected to IPA to determine both specific markers' performance and identification of known disease markers. Top-ranked functions were determined along with those genes with known disease biomarker functions. Disease biomarkers were also mapped in GSNs to determine key transcriptional regulators and relevance to our previous whole genome expression signature analyses. Genes with known functions and/or disease biomarker ability were placed into key marker subsets for each ENM. To ascertain whether the sub-chronic *in vitro* markers for MWCNT-exposed cells correlated with whole lung expression values in aspiration-exposed mice, *in vitro* data were correlated to a publically available dataset (www.mwcnttranscriptome.org).

Key marker gene subsets and disease markers were validated using rt-qPCR and Western blot protein expression using previously described methods. Briefly, qPCR was performed on cDNA using ABI 2720

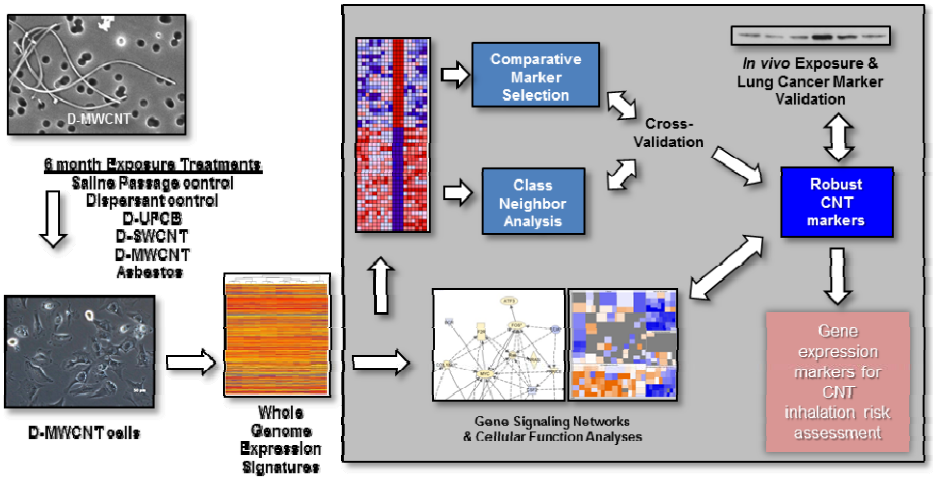

Figure 1: *In vitro* sub-chronic exposure and toxicogenomic screening model to identify nanomaterial specific exposure and lung cancer markers.

ThermoCycler. Expression values were determined using -$2^{\Delta\Delta ct}$. Next, protein was isolated from $5x10^5$ lysed cells and levels determined by SDS-PAGE and chemiluminescence procedures [7,8].

3 RESULTS

3.1 CNT-exposed Cells Display an Aggressive Neoplastic-like Transformation Phenotype

SWCNT and MWCNT sub-chronic exposure displayed an aggressive neoplastic-like transformation effect compared to asbestos and ultrafine carbon black in human small airway epithelial cells (Figure 2) [8]. Both CNT and ASB cells displayed significantly increased mitochondrial metabolism and live cell number at chosen time points. UFCB showed significant proliferation decline. Next, CNT cells showed significantly enhanced invasion and migration potential while ASB cells displayed only a moderate significant increase in invasion. Third, CNT cells formed significantly more colonies in soft agar indicating attachment-independent tumor-like growth ability.

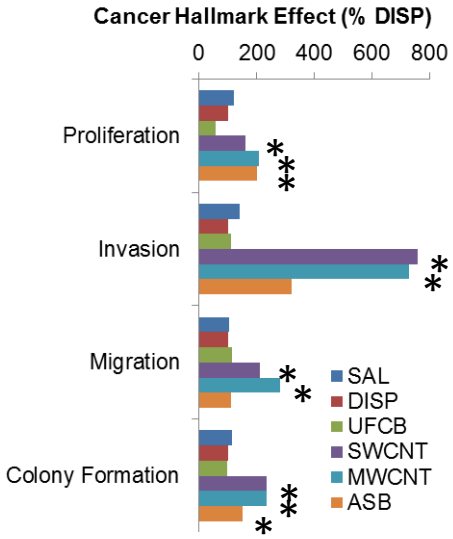

Figure 2: Cancer hallmark phenotype asssessment of sub-chronically ENM exposed SAECs. * represent differences from dispersant control ($p < 0.05$).

3.2 Proto-oncogene Signaling in CNT-Exposed Cells Versus Pro-inflammatory Signaling in ASB-Exposed Cells

CNT-exposed SAECs displayed a high number of DEGs associated with cell proliferation, death, movement and devleopment. ASB cells showed large changes in similar functions but were all associated with known pro-inflammatory signaling (data not shown). UFCB showed decreased expression in proliferation, assembly, and function correlating with its slow proliferation. Large changes in lipid metabolism was a distinct CNT cell signature characteristic, while inflammation was distinct for ASB cells. Based on increased cancer cell hallmark activity, GSN analysis for pro-cancer signaling showed that CNT exposure caused a protooncogene (*MYC*, *PPARG*, *CASP8*, and *COL18A1*) centered network while ASB exposure resulted in an inflammation (*IL1B, CCL2* and *SPI1*) associated network (data not shown).

3.3 Particle-Specific CNT Markers *vs.* ASB Markers

Robust and distinct ENM specific gene marker sets were found for *in vitro* exposed SAEC (Table 1). Both SWCNT and MWCNT markers were associated with lipid metabolism and cancer, while ASB and UFCB centered on inflammatory response and senescence, respectively. Functions associated with each particle's small gene marker subset were predictive of the entire whole genome signature and exposed cell phenotype. Biomarker Analysis identified known lung and other cancer markers (eg. *MYC*, *PPARG*, *COL18A1*) in CNT-treated cells which differed from inflammation-associated cancer markers (*IL1B*, *SPI1*) in ASB cells. Furthermore, several *in vitro* MWCNT (*OLFMF2A*, *HMGCR*, *FABP3*) and SWCNT (*ALDH3A2*, *C1QBP*) markers correlated with *in vivo* whole lung gene expression from MWCNT-aspirated mice [14] indicating the potential use of this *in vitro* screening model for risk assessment.

3.4 Potential CNT Tumor-Associated Biomarkers

Several genes in the top 100 ranked genes for SWCNT, MWCNT and ASB cells are known lung cancer and/or other cancer markers. mRNA and protein expression validated (data not shown) that SWCNT exhibited over-expressed *AKR1B10*, *C1QBP*, *PRDX1*, and *USP22*. MWCNT cells over-expressed *HMGCR*, *SKP2*, and *WIP1* while ASB cells over-expressed *IL1B* and *SPI1*. Correlation analysis of several *in vitro* MWCNT markers to whole lung gene expression for aspirated mice [14] using the same MWCNT particle showed that *HMGCR* and *MDK* exhibited high correlation at ≥ 1 time point at moderate to high aspirated doses (data not shown).

4 DISCUSSION

Sub-chronic *in vitro* exposure of SAECs to SWCNT and MWCNT resulted in an aggressivie neoplastic-like transformation phenotype that differed from asbestos, UFCB and passage control exposed cells. Evaluation of GSNs suggested that both sets of CNT- treated cells exhibited pro-cancer GSNs involving several well-established protooncogenes (*MYC*, *COL18A1*, *CDKN2A*),

Particle	Gene Biomarker	Major Functions
UFCB	**KIAA1841, RUTBC2, AFG3L1, CD160**, DCUN1D4, RGL2, RAB27A, AAK1, TSNAX, C3orf23	Endocytosis, Transcriptional Regulation
SWCNT	**ELAC2, PMP22, INSIG1, GABRA3, ALDH3A2,** B3GALT6, IGFBP3, PAF1, MEGF8, GPR108	Membrane trafficking, lipid metabolism, proliferation
MWCNT	**OLFML2A, HMGCR, COPS6, FABP3, SLC25A17,** GSTK1, FASTK, RASSF4, TFCP2L1, STX8, MDK	Peroxisome/endosome function, proliferation, lipid metabolism
ASB	**FCER1G, IL1B, FCGR2A, IL10RA, HCK, CLEC7A, NCF2, ATP2A3, SPI1,** IL7R	Immune & Inflammatory Response

Table 1: Top ranked Specific Gene Biomarker Subsets in Sub-Chronically Exposed Human Small Airway Epithelial Cells. Bold and normal text indicate over- and under-expression, respectively.

while ASB cell pro-cancer GSN was dominated by pro-inflammatory signaling (*IL1B*, *SPI1*, *CCL2*).

Comparative marker selection with cross validation provided a robust biosignature for UFCB-, CNT- or asbestos-exposed SAEC. Top–ranked biomarker subsets and their functions correlated with top-ranked functions from the whole genome expression analysis. CNT-exposed SAEC biomarkers were associated with membrane, lipid metabolism, proliferation and cancer, while ASB biomarkers possessed an inflammation response signature. Recent studies have suggested that CNT pulmonary exposure results in lipid-specific signatures and promotes tumorigenesis while inflammation promotion of lung fibrosis and tumorigenesis is well-established [2,15]. In addition, this 'omics' signature approach identified several known lung cancer genes within each top-ranked gene subset. *MYC*, *COL18A1*, *USP22*, *C1QBP*, and *WIP1* in CNT cells all have known clinical associations with lung and other cancers while *SPI1* in ASB cells was recently identified as a lung cancer marker. This suggests that comparing multiple ENMs using 'omic' signatures is a feasible strategy for identifying smaller subsets of biomarker genes to assist in not only exposure, but early disease detection.

Implimentation of *in vitro* 'omics' screening strategies, once compared to applicable *in vivo* datasets, are key parts of several intense research initiatives (eg. EPA ToxCast; NCI Cancer Genomics) and hold promise as a high-throughput screening tools for identification of exposure and disease biomarkers for comparative risk assessment.

At present, studies to detemine U.S. workplace CNT exposures and development of a reliable set of inhalation exposure biomarkers is underway at NIOSH [16,17]. Our proposed CNT, ASB and UFCB biomarker subsets require further independent class prediction analysis and validation in appropriate 'bridge' models, *in vivo* exposure models and collected human samples. These particle-specific markers may serve useful as improvements in clinical techniques (blood genetic screenings, personalized medicine) become implemented [18]. Our findings suggest that using phenotype and 'omics' screening assessments against numerous ENMs in a sub-chronic *in vitro* exposure model can potentially aid in identifying ENMs of concern for human health and sets of biomarkers to assist in early disease detection [12].

REFERENCES

[1] K. Donaldson and C. Poland. Swiss Med. Wkly. 142, w13547, 2012.

[2] L. Sargent, D.W. Porter, L. Staska, A.F. Hubbs, D.T. Lowry, et al. Part Fibre Toxicol., 11, 3, 2014.

[3] A. Takagi, A. Hirose, M. Futakuchi, H. Tsuda and J. Kanno. Cancer Sci, 103, 1440-44, 2012.

[4] H. Nagai, Y. Okazaki, S.H. Chew, N. Misawa, Y. Yamashita, et al. PNAS, 108, E1330-38, 2012.

[5] D.W. Porter, A.F. Hubbs, R.R. Mercer, N. Wu, M.G. Wolfarth, et al. Toxicology, 269, 136-47, 2010.

[6] R.R. Mercer, J.F. Scabilloni, A.F. Hubbs, L.A. Battelli, W. McKinney, et al. Part. Fib. Toxicol. 10, 33, 2013.

[7] L. Wang, S. Luanpitpong, V. Castranova, W. Tse, Y. Lu, V. Pongrakhannon and Y. Rojanasakul. NanoLetters, 11, 2796-803, 2011.

[8] L. Wang, T.A. Stueckle, A. Mishra, R. Derk, V. Castranova and Y. Rojanasakul. Nanotoxicology, 8: 485-507, 2014.

[9] L. Guo, Y.W. Wan, J. Denvir, D. Porter, M. Pacurari, M. Wolfarth, V. Castranova, Y. Qian. J Toxicol Environ Health A, 75, 1129-53, 2012.

[10] J. Teeguarden, B. Webb-Robertson, K. M. Waters, A.R. Murray, E. Kisin, et al. Tox. Sci, 120, 123-35, 2010.

[11] I. Iavicoli, V. Leso, M. Manno and P.A. Schulte. J. Nanopart. Res, 16, 2302, 2014.

[12] S.C. Gordon, J.H. Butala, J.M. Carter, A. Elder, T. Gordon, et al. Regul. Toxicol. Pharmacol. 68, 305-11, 2014.

[13] T.R. Golub, D.K Slonin, P. Tamayo, C. Huard, M. Gaasenbeek, et al. Science, 286, 531-7, 1999.

[14] B.N. Snyder-Talkington, J Dymacek, D.W. Porter, M.G. Wolfarth, R.R. Mercer, et al. Toxicol Appl Pharmacol. 272, 476-89, 2013.

[15] Tyurina, E.Kisin, A. Murray, V. Tyurin, V. Kapralova et al. ACS Nano 5, 7342-53, 2011.

[16] M. Dahm, D.E. Evans, M.K. Schubauer-Berigan, M.E. Birch and J.E. Fernbeck. Ann Occup Hyg. 56, 542-56, 2012.

[17] A. Erdely, M. Dahm, B.T. Chen, P. Zeidler-Erdely, J. Fernback, et al. Part.Fib. Toxicol. 10, 53, 2013.

[18] A. Newman, S. Bratman, J. To, J. Wynne, et al. Nat. Med., 3519, 2014.