

Research Article

Gene Expression Profiles in Peripheral Blood Mononuclear Cells of Chinese Nickel Refinery Workers with High Exposures to Nickel and Control Subjects

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Abstract

Background: Occupational exposure to nickel (Ni) is associated with an increased risk of lung and nasal cancers. Ni compounds exhibit weak mutagenic activity, alter the cell's epigenetic homeostasis, and activate signaling pathways. However, changes in gene expression associated with Ni exposure have only been investigated *in vitro*. This study was conducted in a Chinese population to determine whether occupational exposure to Ni was associated with differential gene expression profiles in the peripheral blood mononuclear cells (PBMC) of Ni-refinery workers when compared with referents.

Methods: Eight Ni-refinery workers and ten referents were selected. PBMC RNA was extracted and gene expression profiling was conducted using Affymetrix exon arrays. Differentially expressed genes (DEG) between both groups were identified in a global analysis.

Results: There were a total of 2,756 DEGs in the Ni-refinery workers relative to the referents [false discovery rate (FDR) adjusted $P < 0.05$] with 770 upregulated genes and 1,986 downregulated genes. DNA repair and epigenetic genes were significantly overrepresented ($P < 0.0002$) among the DEGs. Of 31 DNA repair genes, 29 were repressed in the Ni-refinery workers and 2 were overexpressed. Of the 16 epigenetic genes, 12 were repressed in the Ni-refinery workers and 4 were overexpressed.

Conclusions: The results of this study indicate that occupational exposure to Ni is associated with alterations in gene expression profiles in PBMCs of subjects.

Impact: Gene expression may be useful in identifying patterns of deregulation that precede clinical identification of Ni-induced cancers. *Cancer Epidemiol Biomarkers Prev*; 22(2); 261–9. ©2012 AACR.

Introduction

Nickel (Ni) compounds are ubiquitous pollutants in occupational and environmental settings. In the environment, Ni is found as a contaminant in the air and drinking water, or as either a constituent or contaminant in a variety of foods including chocolate, peanuts, and grains (1). Environmental exposure to Ni also occurs by dermal contact with industrial and commercial products containing Ni alloys including jewelry, household, and cooking

utensils, orthodontic appliances, dental tools, orthopedic implants, and batteries (1). Occupational exposure to Ni occurs by inhalation in the mining, refining, alloy production, electroplating, and welding industries (2–4).

Human exposure to Ni-containing products can result in a variety of adverse health effects. Ni allergy is one of the most common causes of contact allergic dermatitis (5). Ni is of great environmental concern, as epidemiologic studies of Ni compounds from occupationally exposed populations have reported an increased incidence of lung and nasal cancers and elevated risks of acute respiratory syndromes, most clearly shown in Ni refinery workers (2, 3). Ni compounds have been classified as class 1A human carcinogens by the International Agency for Research on Cancer (6).

To date, the precise molecular mechanism(s) of Ni carcinogenesis has not been clearly defined. Using animal and *in vitro* cell models, Ni compounds have been shown to promote the generation of reactive oxygen species (7); interact directly or indirectly with nucleic acids and cause genotoxic damage (8); activate signaling pathways such as phosphoinositide 3-kinase/AKT (9), hypoxia-inducible factor-1 α (10), NF- κ B (11–13), activator protein (12, 13), and nuclear factor of activated T cells (14). In addition,

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chromatin structural alterations and changes in epigenetic marks have been implicated in Ni carcinogenesis (15–20). Previous studies have shown that *in vitro* exposure to Ni induces global DNA hypermethylation and can perturb global and gene specific levels of DNA methylation and posttranslational histone modifications. Ni-induced changes in posttranslational histone modifications can include loss of acetylation on histones H2A, H2B, H3, and H4, as well as increases in histone H3K9 dimethylation, H3K4 trimethylation, and H2A and H2B ubiquitylation (15–18).

The changes in gene expression patterns that occur in human populations occupationally exposed to Ni compounds have not been previously studied. Gene expression may be useful in identifying patterns of deregulation that precede clinical identification of Ni-induced cancers. Several studies have suggested that gene expression data from peripheral blood mononuclear cells (PBMCs) can provide valuable information about exposure levels (21–25). The use of PBMCs has tremendous potential in a therapeutic setting, as the use of blood as a surrogate for the primary tissue of interest greatly facilitates sample collection and analysis. Here, we have compared the differential gene expression profiles between subjects with occupational exposure to Ni (Ni-refinery workers) and referent subjects with low-level environmental exposures to identify gene expression changes associated with exposure to high levels of Ni.

Materials and Methods

Study site and subject recruitment

This study was conducted among Ni refinery workers in Jinchang, China, and local residents in Gansu, China. Jinchang City, where the Ni refinery is located, is within the providence of Gansu. Study selection was based on the fact that the subjects from the high exposure group were workers of the Ni refinery and the referent subjects were local residents of the same providence, but with no known occupational exposure to Ni. The human subject protocol for this study was approved by the Institutional Review Boards of both the New York University School of Medicine and the Lanzhou University School of Public Health. Written informed consent was obtained from all participating subjects.

The subjects for this study were randomly selected from a study population previously described (20). Eighteen healthy male subjects between 42 and 56 years of age were recruited; subjects diagnosed with chronic diseases, including cancer, were excluded. Eight subjects who had high occupational exposure to Ni having worked for at least 1 year at a Ni refinery in Jinchang, China in the flash smelting workshop where sulfidic Ni ores were processed and 10 referent subjects from residents in Gansu, China who were maintenance or office workers, with no reported occupational exposure to Ni, were also selected. Ni-refinery workers are exposed to ambient Ni concentrations as high as 1 mg/m³. Referent subjects are exposed

to ambient air concentrations of 204.8 ± 268.6 ng/m³ of Ni. This study was conducted to determine whether Ni exposure was associated with differential gene expression profiles in the PBMCs of subjects.

Sample collection and handling

Blood collection, handling, and PBMC isolation for this study was conducted as previously described (20). Fifty milliliter of urine was also collected from each subject. The isolated PBMC pellet was suspended in TRIzol (Invitrogen) and urine samples were stored at –80°C and hand-carried frozen on dry ice to New York University.

RNA isolation, amplification, and hybridization

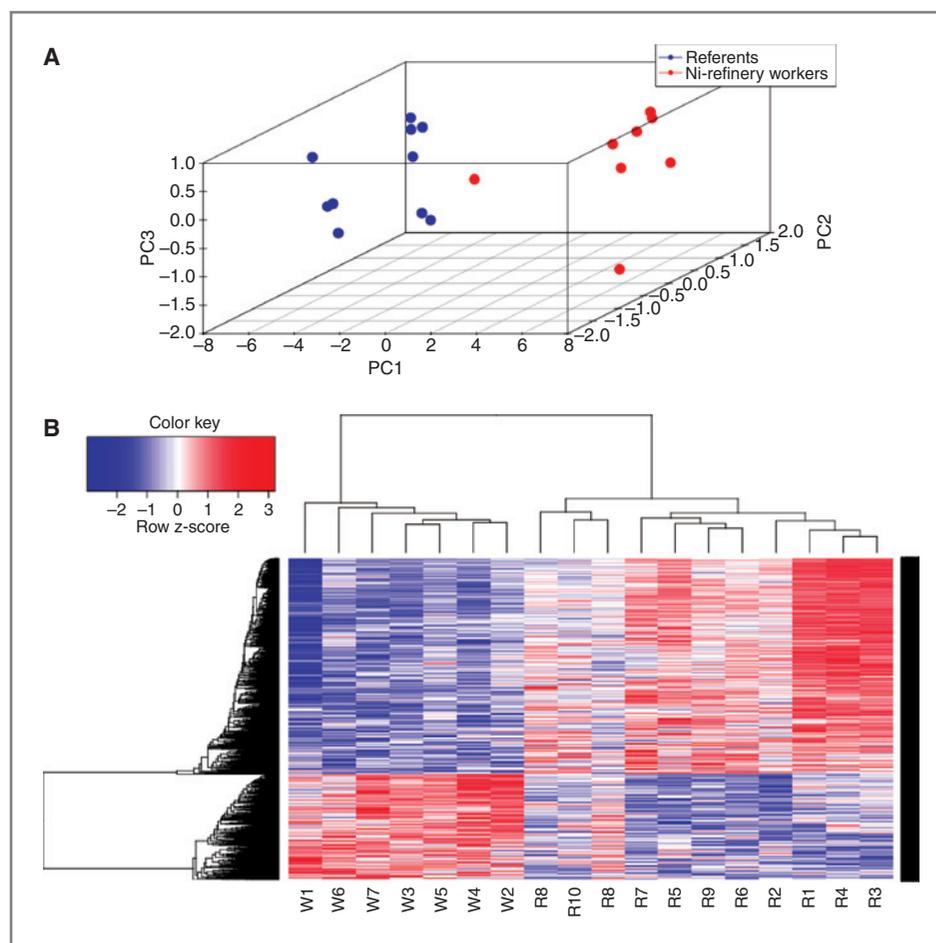
Total RNA was extracted from each sample according to the TRIzol (Invitrogen) manufacturer's protocol. cRNA probes were synthesized and labeled using GeneChip Whole Transcript Terminal Labeling Expression Kit (Affymetrix) and were subjected to hybridization with GeneChip Human Gene 1.0 ST Array (Affymetrix) that contains 28,869 well-annotated genes. Hybridization and scanning of the arrays were conducted using a standard procedure.

Data analysis to identify differentially expressed genes

Gene expression analyses were conducted using R. Gene expression data were imported and normalized in batches using the Affymetrix package version 1.30.0 in R 2.13.2 GUI 1.42 Leopard build 32-bit and robust multichip average (26, 27). Samples were then batch normalized and log-transformed using an empirical Bayes approach with the R package COMBAT run through SVA 3.0.2 in R 2.14.0 GUI 1.42 Leopard build 32-bit (28). Significance of gene expression changes between the exposed and referent populations was assessed using a gene-wise linear model approach with LIMMA 3.8.3, which uses an empirical Bayes approach to generate moderated *t* statistics by taking into account the standard errors and estimated log-fold changes (29). *P* values were subjected to FDR (false discovery rate) correction for multiple hypothesis testing and adjusted *P* values with *P* < 0.05 were considered significant (30). Genes that met significance thresholds via this approach were included in our study set for further analysis and are referred to as differentially expressed genes (DEG) or the "study set." All microarray data is MIAME compliant and the raw data has been deposited in NCBI's Gene Expression Omnibus, and assigned Series accession number GSE40392.

To investigate the biologic relevance of the genes in our study set, we assessed the level of representation of previously established molecular signatures. Using the molecular signatures database (Broad Institute Gene Set Enrichment Analysis) the DEG were evaluated for overlap with gene sets related to position, biologic function, and disease states. This analysis led to the identification of a gene set involved in nasopharyngeal carcinoma (NPC) and further analysis was conducted using this

Figure 1. Gene expression profiles of PBMCs from Ni-refinery workers when compared with referents. A, principal components analysis revealed distinct separation between Ni-refinery workers and referents. Red circles: Ni-refinery workers; blue circles: referents, subjects with environmental exposure. B, hierarchical clustering analysis of 2,756 DEGs (adjusted $P < 0.05$) in which samples were sorted based on the similarity of gene expression revealed distinct separation between Ni-refinery workers (designated as W in figure) and referents (designated as R).



experimentally generated set (31). DNA repair genes were also investigated based on their previously established link with NPC and were tested for their overrepresentation in the DEG set using a hypergeometric distribution as previously described (32). On the basis of this overlap, a list of 31 DNA repair genes were identified in the DEG and used in further analysis. Epigenetic genes were investigated based on Ni's known role as an epigenetically active agent and were analyzed in the same manner as the DNA repair genes.

Heat maps and principal components analysis (PCA) plots were generated using R. The heat map in Fig. 1 was generated using the study set. The heat map in Fig. 2 was generated based on the intersection of the study set and genes downregulated in NPC (31). The heat map in Fig. 3A was generated by taking the intersection of the study set and an extensive list of DNA repair genes to generate a gene set of significantly changed DNA repair genes (33–35). The heat map in Fig. 3B was generated by taking the intersection of the study set and a compiled list of epigenetic related genes (36, 37). Correlation analysis for heat maps was conducted using Pearson correlation analysis and hierarchical clustering along both axes was conducted using the average method.

Real-time quantitative PCR

Total RNA was extracted from each sample using TRIzol Reagent (Invitrogen), and converted to single

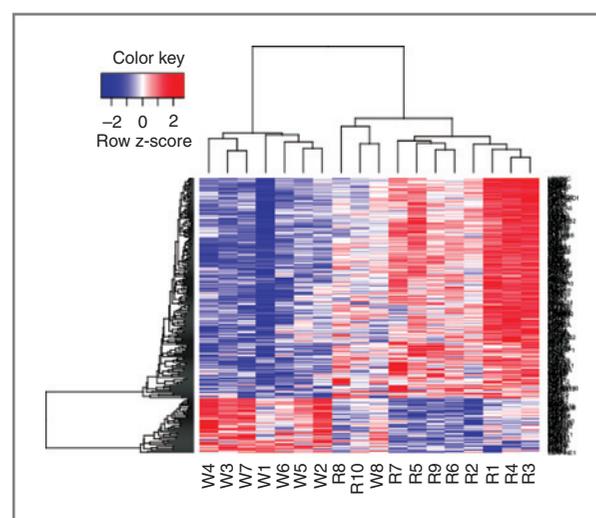


Figure 2. Heat map of genes associated with NPC. See Supplementary Table S2 for full list of genes.

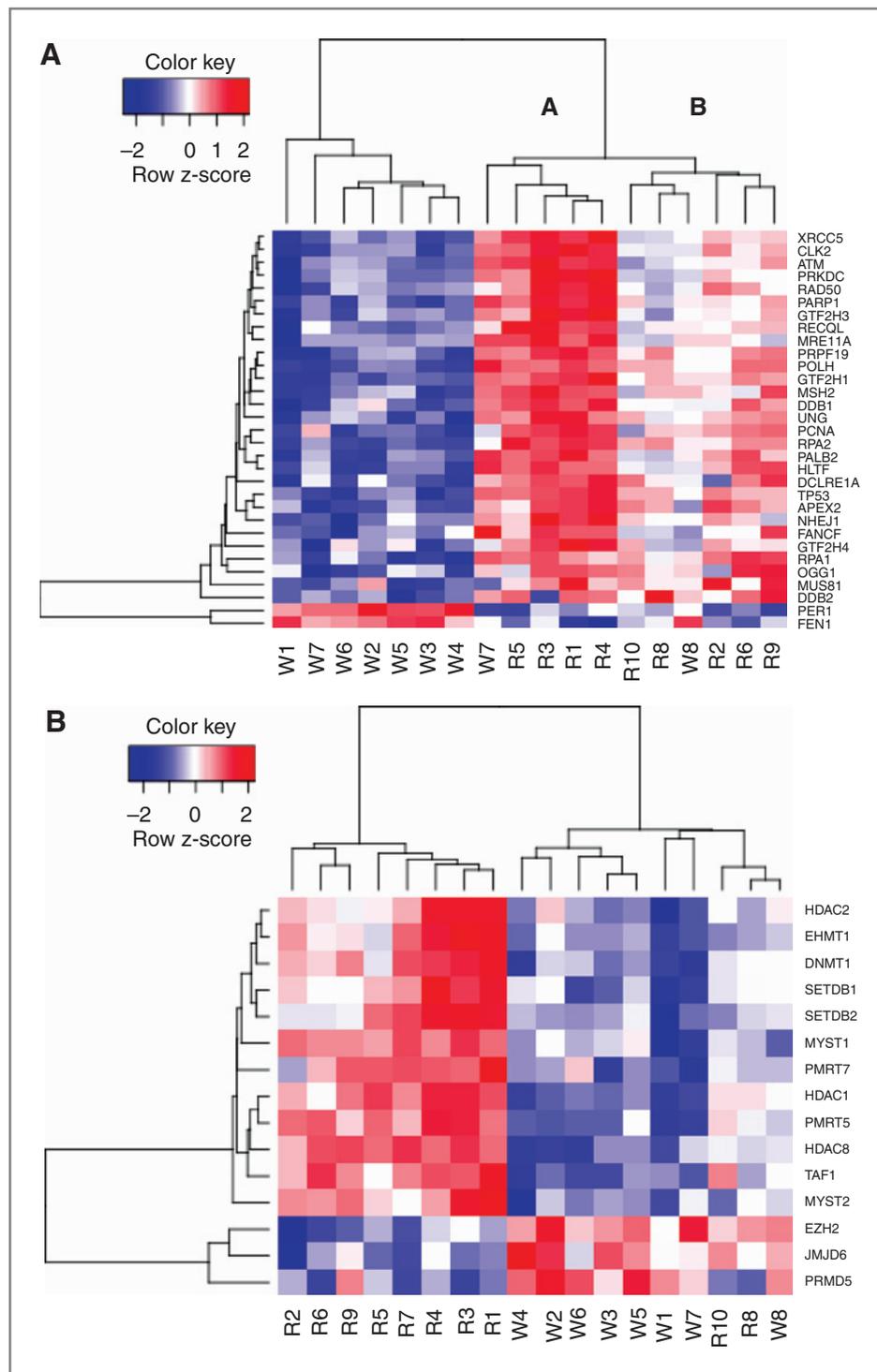


Figure 3. A, heat map of DNA repair genes. DNA repair genes include the following from top to bottom: *XRCC5*, *CLK2*, *ATM*, *PRKDC*, *RAD50*, *PARP1*, *GTF2H3*, *RECQL*, *MRE11A*, *PRPF19*, *POLH*, *GTF2H1*, *MSH2*, *DDB1*, *UNG*, *PCNA*, *RPA2*, *PALB2*, *HLTF*, *DCLRE1A*, *TP53*, *APEX2*, *NHEJ1*, *FANCF*, *GTF2H4*, *RPA1*, *OGG1*, *MUS81*, *DDB2*, *PER1*, and *FEN1*. See Supplementary Table S3 for the list of genes and associated *P* values. B, heat map of epigenetic genes. Epigenetic genes include the following from top to bottom: *HDAC2*, *EHMT1*, *DNMT1*, *SETDB1*, *SETDB2*, *MYST1*, *PMRT7*, *HDAC1*, *PMRT5*, *HDAC8*, *TAF1*, *EHMT2*, *MYST2*, *EZH2*, *JMJD6*, and *PRMD5*. See Supplementary Table S4 for the list of genes and associated *P* values.

stranded cDNA using Superscript III (Invitrogen). Quantitative real-time PCR (qRT-PCR) analysis was conducted using SYBR green PCR system (Applied Biosystems) on ABI prism 7900HT system (Applied Biosystems). All qRT-PCR reactions were conducted in triplicate. Because of lack of sufficient biologic material per sample, eight different RNA samples from the same study cohort that were

not part of the original microarray analysis were used for the qRT-PCR analysis. Quantitative real-time PCR was conducted on *CCL20*, *IL-6*, and *FOSB* (upregulated in Ni-refinery workers) and *CX3CR1* and *MNDA* (downregulated in Ni-refinery workers) in four Ni-refinery workers and four referents. The data is presented as the absolute fold change in gene expression of Ni-refinery worker

samples relative to the referents. The expression of the target genes was first normalized to the expression of the house-keeping gene (*GADPH*) for the referent and Ni-refinery worker samples. Then the mean of the Ni-refinery worker samples was normalized to the mean of the referent samples.

Measurement of urinary nickel, cotinine, and creatinine

Urinary Ni was used to index the individual personal exposure to Ni and was analyzed for all study subjects by inductively coupled plasma mass spectroscopy (Elan DRCII, Perkin Elmer; ref. 38). Urinary cotinine, a major metabolite of nicotine and a valid biomarker of environmental tobacco smoke, was measured in each subject to confirm smoking status and control its potential confounding effects. Urinary cotinine was measured using a cotinine direct ELISA kit (Immunalysis; ref. 39). Urinary creatinine was measured to adjust the Ni and cotinine levels in urine samples. Urinary creatinines were determined using the creatinine incorporating dynamic stabilization technology assay kits (Fisher Scientific) according to the standard procedure.

Statistical analysis

Differences in age, self-reported smoking data (number of smokers, number of years smoking, and cigarettes per day smoked for smokers), urinary cotinine, and urinary Ni among groups were compared by 2-sample Student's *t* test. Differences in number of smokers and nonsmokers between exposure groups were compared by χ^2 test. All *P* values were 2 sided, with *P* < 0.05 considered statistically significant. Statistical analyses were conducted in S-Plus statistical analysis software (TIBCO Software Inc.).

Results

In the present study, a total of eighteen subjects, eight Ni-refinery workers and ten referents, were selected from a previously described study to determine whether occupational exposure to Ni induces alterations in gene expression patterns in PBMCs (20). As shown in Table 1, no significant difference was found between Ni-refinery workers and referents, with respect to age, self-reported data on smoking habits, or urinary cotinine levels. As expected, urinary Ni was elevated in Ni-refinery workers (6.86 ± 1.60) when compared with referents (3.41 ± 3.30 ; *P* = 0.0119). Although a large percentage of subjects reported that they were smokers in both groups, it is widely accepted that the Ni concentrations in blood plasma and urine are similar among smokers and nonsmokers (40).

To characterize the differences in gene expression profiles in PBMCs of subjects with occupational exposure compared with referents, we analyzed the gene expression profiles of PBMCs from both Ni-refinery workers and referents using Affymetrix Human Gene 1.0 ST Array containing 28,869 well-annotated genes. There were a total of 2,756 DEGs in the Ni-refinery workers relative to the control subjects (FDR-adjusted *P* < 0.05) with 770 upregulated genes and 1,986 downregulated genes. A total of 2,158 genes in samples from Ni-refinery workers displayed a greater than 1.25 fold change as compared with referents. When the cut-off threshold was increased to 1.50 and 2.0 fold change, the numbers decreased to 578 and 58, respectively. PCA of the microarray data revealed a clear separation between Ni-refinery workers and referents (Fig. 1A). Similar results were observed with a hierarchical clustering analysis of DEG (adjusted *P* < 0.05) in which samples were sorted based on the similarity of gene expression (Fig. 1B). To validate the microarray results,

Table 1. Characteristics of the Ni-refinery workers and referents selected to assess gene expression profiles in PBMCs

Parameter	Referent subjects	Ni-refinery workers	<i>P</i>
	Mean \pm SD	Mean \pm SD	
Age	43.90 \pm 8.84	45.50 \pm 5.45	0.6441
Smoking (self-reported)			
Smokers [N/(%)]	[7/70%]	[6/75%]	0.8262
Smoking years	10.70 \pm 10.91	12.75 \pm 10.69	0.6943
Cigarettes/day	11.70 \pm 15.35	9.25 \pm 9.44	0.6833
Urinary cotinine (μ g/g creatinine)	8,115.82 \pm 10,345.48	9,034.08 \pm 13,552.52	0.8767
Urinary Ni (μ g/g creatinine)	3.41 \pm 3.30	6.86 \pm 1.60	0.0119
Median	2.37	6.32	
Min	1.19	5.23	
Max	12.58	9.44	
10 th percentile	1.19	5.23	
25 th percentile	2.30	5.37	
75 th percentile	3.25	8.38	
90 th percentile	12.58	8.38	

Table 2. Validation of microarray results by quantitative real-time PCR

Gene name	Gene symbol	Microarray	qRT-PCR
<i>Chemokine (C-C motif) ligand 20</i>	<i>CCL20</i>	4.20	8.79
<i>Interleukin-6 (interferon, beta 2)</i>	<i>IL6</i>	4.02	4.8
<i>FBJ murine osteosarcoma viral oncogene homolog B</i>	<i>FOSB</i>	2.14	1.75
<i>Chemokine (C-X3-C motif) receptor 1</i>	<i>CX3CR1</i>	0.26	0.51
<i>Myeloid cell nuclear differentiation antigen</i>	<i>MNDA</i>	0.35	0.40

NOTE: Data is represented as absolute fold-change values for the exposed group (Ni-refinery workers) relative to the referents for both the microarray and qRT-PCR.

qRT-PCR was conducted in four Ni-refinery workers and four referents on *CCL20*, *IL-6*, *FOSB*, (upregulated in Ni-refinery workers), *CX3CR1* and *MNDA* (downregulated in Ni-refinery workers). As shown in Table 2, the up- or downregulated patterns for these 4 genes obtained from qRT-PCR were similar to those in the microarray study. In comparing the fold-change values between the microarray data and the qRT-PCR data (Table 2), we see the changes are comparable in that both sets of data reflect fold changes occurring in the same direction with similar magnitudes. It is important to note that this comparison is occurring across different types of data, where the fold-change values for the microarray data are qualitative and the fold-change values for the qRT-PCR are quantitative, and therefore, we do not expect such values to be exactly the same. Thus, the match in direction and the similarity in magnitude constitute consistency in the findings.

To assess the biologic relevance of the DEG, (adjusted $P < 0.05$) those genes exhibiting more than a 1.15 fold-change difference in gene expression (Supplementary Table S1), including a total of 2,734 genes, with 756 upregulated and 1,978 downregulated genes, were to identify the functional categories that were significantly overrepresented ($P < 0.05$) in Ni-refinery workers. Gene families overrepresented with the list of genes differentially expressed between Ni-refinery workers and referents are listed in Table 3. The largest group of gene families, in terms of number of genes, was transcription factors, followed by protein

Table 3. Gene families represented with list of genes differentially expressed between Ni-refinery workers and referents ($P < 0.05$)

Gene family	Number of genes
Oncogenes	46
Tumor suppressors	21
Transcription factors	233
Cytokines/growth factors	37
Protein kinases	98
Translocated cancer genes	46
Cell differentiation markers	82
Homeodomain protein	12

kinases, cell differentiation markers, oncogenes, translocated cancer genes, tumor suppressors, and homeodomain proteins, respectively.

Analysis of molecular signatures revealed that the DEG had significant levels of overlap with gene sets categorically classified as cancer modules (i.e., NPC), cancer neighborhoods (i.e., *BRCA1* network), and genes related to chemical and genetic perturbations. Our Ni exposure related gene set showed highly significant ($P < 0.00E + 00$) overlap with a gene set composed of genes downregulated in NPC tumors (31), where 253 genes from the study set were present in the NPC set (Supplementary Table S2). Of these 253 genes, 51 were upregulated and 202 were downregulated. Cluster analysis of the genes related to NPC revealed cluster formation that is consistent with the full study set (Fig. 2).

DNA repair genes were significantly overrepresented among the DEG 2-fold ($P < 0.0002$). Of 31 DNA repair genes, 29 were repressed in the Ni-refinery workers and 2 were overexpressed (Supplementary Table S3). Cluster analysis with DNA repair genes yielded 2 primary clusters that largely correspond to the occupational and referent groups, but unlike the cluster formation in the study set, there were 2 main subbranch clusters within the referent population, where the left subbranch (A) was characterized largely by overexpression and the right subbranch (B) was characterized by expression levels near the mean (Fig. 3A). Epigenetic genes were also significantly overrepresented in the DEG by 2-fold ($P < 0.02$). Of these 16 epigenetic genes, 12 were repressed in the Ni-refinery workers and 4 were overexpressed (Supplementary Table S4). Similar results were observed with a hierarchical clustering analysis of epigenetic genes in which samples were sorted based on the similarity of gene expression (Fig. 3B).

Discussion

Ni is a well-established human carcinogen associated with an increased risk of lung and nasal cancers. However, the molecular mechanisms and changes in gene expression that mediate Ni's toxicity and carcinogenicity are not well understood. In the present study, we found that differential patterns of gene expression in PBMC exist among Ni-refinery workers compared with referent

subjects, who are only environmentally exposed to Ni. There were a total of 2,756 DEGs in the Ni-refinery workers relative to the control subjects (FDR adjusted $P < 0.05$) with 770 upregulated genes and 1,986 downregulated genes. Although various mechanisms for the carcinogenesis of Ni compounds have been postulated, the changes in gene expression associated with occupational exposure to Ni have not been previously investigated. Here we have found that the function of the majority of the Ni-induced and repressed genes by *in vivo* exposure to Ni were related to cytokine–cytokine interaction and chemokine signaling (*IL1A*, *CCL20*, *CCR2*, *CX3CR1*, *IL6*, *IL1RN*, *TNFSF10*, *IL8RB*, and *IL8RA*). Of particular interest is *IL-1*, a cytokine known to be elevated in many tumor types and which has been implicated as a factor in tumor progression by its ability to increase the expression of metastatic, angiogenic, and growth factor genes (41). Interestingly, *IL1A* was the most upregulated gene in the PBMCs of the Ni-refinery workers. These results suggest that high exposure to Ni compounds was associated with increased expression of cancer related genes. In this case, the high expression of *IL1A* and other genes in Ni-refinery workers suggests that these genes should be further studied to identify their role in Ni-induced adverse health effects.

Moreover, the genes aberrantly expressed in this study share a significant similarity with those aberrantly expressed in NPC tumors as described in Dodd *et al.* (2006). Nasal and sino-nasal cancers have been historically established as an endpoint of occupational Ni exposure (2, 3), and the similarity in gene deregulation between the refinery workers in our study and NPC tumors, suggests that gene expression profiling can be used to link human *in vivo* Ni exposures to cancerous endpoints by identifying gene expression patterns that precede clinical presentations of cancer. Although the two cancer endpoints in discussion here are not synonymous, their anatomical proximity and the significance of our P value merits further consideration. While genes from NPC were significantly overrepresented in our study, there was not a complete overlap and the direction of deregulation was only partially consistent with the Dodd study set. These results likely reflect the similar endpoints, cancers of the nasal and nasopharynx regions, but also point toward the different etiologies likely involved in these related but distinct cancers. The Dodd study emphasizes that NPC is often associated with Epstein-Barr virus and that factor is not one that we would expect to be represented in our study. Both etiologies show a strong involvement of the DNA repair genes, but involve different groups of genes with only *UNG*, *MSH2*, *PRKDC*, and *PCNA* in common. The Dodd and colleagues' study found DNA repair genes to be upregulated, whereas we observed that DNA repair genes were largely repressed in the exposed population. The repression of DNA repair genes observed in our study may be explained by Ni's role as an epigenetically active agent that can actively silence genes. The repression of DNA repair genes was associated with high levels of exposure, and may be due to Ni-induced silencing, a

process which is supported by the significant overrepresentation of epigenetic genes in the study set, their aberrant expression patterns relative to the referents, and a previous study which showed that occupational exposure to Ni induces consistent changes in histone modifications (15–18, 20, 42). Together, the changes observed in epigenetic regulation and suppression of DNA repair can be detrimental to the genetic stability of cells, lead to increased spontaneous mutations, and promote conditions in which carcinogenesis is favored.

The importance of identifying genes through an *in vivo* format can be highlighted with a comparison of our *in vivo* data and pilot data from an *in vitro* study. We assessed the short-term gene changes associated with PBMC's, exposed to Ni *in vitro* and found that gene changes at the 1.5 fold-change level were strikingly different from those observed in the *in vivo* study. PBMC's were exposed to Ni chloride (NiCl_2) at 1.0, 0.5, and 0.25 mmol/L for 24 hours and single samples were then prepared for gene expression analysis and hybridized to Affymetrix HGU133a.2 arrays. For all 3 treatments, there were 98 genes that changed 1.5 fold (Supplementary Table S5). Of these genes, only 9 were found among the genes that changed 1.5-fold in the *in vivo* study. These genes include: *CD14*, *FUCA1*, *DAPK1*, *HMOX1*, *FGL2*, *IGF6*, *FCGR1B*, *TLR8*, and *CLEC7A*. While the data from the *in vitro* study remains preliminary and was not subjected to statistical analysis due to a lack of replicates, it does suggest that *in vitro* exposures do not accurately replicate the gene expression changes found *in vivo*. Furthermore, the gene changes observed *in vitro* do not share the similarity with NPC that was evident *in vivo*, again indicating the importance of conducting *in vivo* human studies to identify biomarkers that are linked with disease endpoints.

Identification of genes associated with high occupational exposure to Ni will help identify agents that combat the toxic effects of exposure to Ni or can be used as biomarkers for Ni exposure and effects. It is of interest that occupational exposure to Ni causes 29 of 31 DNA repair genes to be suppressed in expression, whereas in the referent group these same genes were upregulated in the left subbranch (Fig. 3A, Branch A) and were expressed near the mean in the right subbranch (Fig. 3A, Branch B). Interestingly, the repression of 6 of the DNA repair genes (*MUS81*, *PALB2*, *XRCC5*, *PRKDC*, *CLK2*, *HLTF*, and *TP53*) found in this study were also found suppressed in the Beas-2B cell line exposed to Ni *in vitro* (43). This same study also found the expression of 6 epigenetic genes (*EHMT1*, *EHMT2*, *PRMT7*, *DETDB1*, *HLTF*, and *JMD6*) repressed as in our study (43). The formation of three primary clusters and 3 types of DNA repair response suggests the presence of a Ni-induced dose–response mechanism. In subjects with occupational exposure to Ni, DNA repair genes were largely repressed, and in the referent population there were 2 types of low-level responses, in the first response type the DNA repair genes were highly upregulated (Fig. 3A, Branch A), whereas in the second there was no distinct response with values near

the mean (Fig. 3B, Branch B). This clear difference between the worker group and branch A of the referent population suggests there may be a dose-response mechanism whereby high exposure leads to the repression of DNA repair genes and low exposure can lead to their over-expression. While the urinary Ni levels for these 2 groups (worker group and branch A of referents) are not significantly different, the means for the 2 groups do reflect this trend. Further study is needed to determine whether the 2 types of low-level response for branches and A and B of the referent population are reflective of a dose-response mechanism or of another factor such as a genetic polymorphism as there was no statistically significant difference in the urinary Ni levels for these 2 groups either. However, it should be noted that urinary Ni may not be a true measure of worker exposure to Ni, as inhaled particulate Ni compounds (i.e., Ni oxides, etc.) may not be cleared from the lungs into the urine for months to years (44, 45). Although Ni-exposure resulted in the differential expression of a large number of genes, these DEGs may not only be Ni-response genes. Future experiments should focus on whether the gene expression changes found in this study are specific to Ni exposure and/or toxicity and determine whether changes are occurring as a result of exposures to other agents found in Ni refineries.

In summary, we have identified many novel changes in gene expression that were different from the immediate response genes identified from previous studies using acute *in vitro* exposure of tissue culture cells to Ni. These genes may be involved in Ni-induced toxicity, carcinogenesis, and can be used as potential biomarkers of exposure to Ni compounds. Furthermore, these changes also show strong similarity with changes observed in NPC,

suggesting that biomarkers of disease may be developed from gene expression studies of PBMCs. Thus, further studies of the mechanism(s) of Ni-induced alterations in gene expression patterns may be a useful tool for identifying patterns of deregulation that precede clinical identification of Ni-induced cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A. Arita, Y. Chervona, Q. Qu, M. Shamy, M. Costa
Development of methodology: A. Arita, H. Sun
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Arita, J. Niu, Q. Qu, N. Zhao, Y. Ruan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Arita, A. Muñoz, Y. Chervona, Q. Qu, H. A. Clancy, M. Shamy
Writing, review, and/or revision of the manuscript: A. Arita, A. Muñoz, Y. Chervona, M. Shamy, M. Costa
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Arita, A. Muñoz, T. Kluz, M. Costa
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