MATERIALS AND METHODS

Subject. Sixteen coke-oven workers who had worked in one of two coke-oven plants for at least one year in the largest steel company in Taiwan voluntarily participated in this study between July-October, 2009. Coke-oven workers regularly work 6 days and take two days off.[1] Thus, we collected their blood and urine samples at two different time points: one was during the pre-shift work on the first day after two days off, and the second one was at the end-of-shift work on the 6th work day. Information about age and smoking status was also collected before the collection of biological specimens. This study was approved by the Institutional Review Boards of KMHU; all study subjects were written informed consent.

Biomarkers in urine. All urine samples were stored at -68°C until analysis. The detailed analytical method is described elsewhere.[2-6] Briefly, aliquot amount of thawed urine was hydrolyzed with β-glucuronidase/sulfatase (Roche Diagnostics Ltd.), purified with a Sep-Pack C18 cartridge (2 g/12 mL, BondElut® C18 HF, Varian), and condensed by dry N2 purge to obtain a 2-ml extract. The extract was analyzed by using high performance liquid chromatography (HPLC, Beckman Coulter Module 126, UK) equipped with a fluorescence detector (Jasco FP-920, Japan) to determine 1-naphthol (1NP), 2-naphthol (2NP), 9-phenanthrol (9PHE) and 1OHP levels. The linearity (expressing as R^2), limit of detection (LOD), reproducibility (expressing as

coefficient of variation (CV)) and mean recovery rate were 0.9982-0.9998, 1.83-47.56 ng/L, 4.02%-7.27%, and 82.97 %-107.85 % respectively. Urinary creatinine was reacted with alkaline picrate and the creatinine-picrate complex was quantified by spectrophotometry (Hitachi U-2000, Japan) using a wavelength of 520 nm. The concentrations of these four hydroxyl-PAHs were presented in units of μmol/mol creatinine.

RNA preparation. Five ml blood sample was drawn from volunteers. WelPrep RNA Stabilizer (Welgeng Biotech, Taipei, TAIWAN) was immediately added to stabilize the whole-blood total RNA in room temperature for 2 hrs and the samples were transferred to our laboratory within one hour. Then, the stabilized blood samples were stored in a -20^{o}C freezer until extraction within 1 month. Total RNA was isolated with RNeasy® Mini Kit (Qiagen, Hilden, Germany) according to manufacturer protocol. The yield and quality of RNA were assessed by spectrophotometry and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Only samples with an A260/A280 between 1.8 and 2.2 and A260/A230 ratio above 1 were eligible for the subsequent array experiment.

Oligonucleotide DNA microarrays. Human oligonucleotide DNA microarrays (Human Whole Genome OneArray™) from Phalanx Biotech Group (Hsinchu, Taiwan) were used. The Human Whole Genome OneArrayTM (HOAv4.3, Phalanx

Biotech Group, Taiwan) contains 32,050 60-mer oligonucleotide probes, including 28,703 probes corresponded to the annotated genes in Unigene v175 and RefSeq database, 2,265 experimentally defined probes and 1,082 control probes.[7,8] The detailed descriptions of the gene array list are available from http://www.phalanx.com.tw/tech_support/gene_lists.html.

Microarray experiment. One-half μg RNA of each sample was amplified by Illumina TotalPrep RNA Amplification Kit according to the manufacturer's instructions (Ambion, Austin, TX). Then, 10 μg of fragmented biotin-labeled cRNA was hybridized on Phalanx Human OneArray™ by Phalanx hybridization buffer at 50^{o}C in oven for 14-16 hrs using the bubble-mixing method. Each sample was hybridized in triplicate; thus, the total 96 chips were used in this study (16 workers × 2 time points × 3 experiments). After non-specific binding targets were washed, the hybridization arrays were conjugated with fluorescent detector of Strepavidin-Cy3. Finally, arrays were dried by centrifugation and scanned by DNA Microarray Scanner (Agilent Technologies, Santa Clara, US). Images from the scanned arrays were quantified using GenePix® Pro 4.0 (Molecular Devices, Sunnyvale, CA).

Qualification and normalization of microarray chips. Spots in each array with foreground median intensity of wavelength 532 nm greater than or equal to that of background median intensity plus 3 folds standard deviation of wavelength 532 nm

were considered as the “Present” flag and included for the further analysis. In order to evaluate the quality of each array in the entire array experiment, three evaluation steps were performed: basic, reproducible, and diagram. In basic step, three parameters, including percentage of “Present” spots among all spots, the average intensity of “Present” spots, and coefficient of variation of intensity for control spots in the entire arrays were all considered. If any two parameters in one array were located outside the 1.5-folds interquartile range (25th-75th) of same parameters for all arrays, that array was excluded. The remaining arrays were then evaluated in reproducible steps which the repeated arrays of the same sample would pass, when their Pearson’s correlation coefficient was larger than 0.95 and “2-fold percentage” was less than 15% (sFig. 4). The “2-fold percentage” was the percentage of probes among all probes in which the ratio of the same probe between two arrays exceeded 2-fold. In the final diagram step, the density plot of repeated arrays was used to examine the intensity profile of each array. An array would pass if the profile was similar to the rest of arrays in the same phenotype groups. When the arrays passed all three steps, the raw intensity of spots were log-2 transformed for subsequent analysis. To adjust the systematic variation of experiments and dye effects, global Lowess normalizations were performed within repeated arrays of the same sample and between the samples. Spot was included for further analysis when it was “Present” in at least one of the qualified arrays.

Statistical analysis. Mann-Whitney U-test was used to compare the differences between pre-shift and end-of-shift (across-the-shift) work of urinary biomarkers. Since 1OHP in urine was recognized as the best surrogate to represent for ambient

coke-oven emission exposure,[1,2,9] we examined the correlation between urinary across-the-shift 1OHP levels and other biomarkers, including 1NP, 2NP, and 9PHE.

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