Third-generation human mitochondria-focused cDNA microarray and its bioinformatic tools for analysis of gene expression

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To facilitate profiling mitochondrial transcriptomes, we developed a third-generation human mitochondria-focused cDNA microarray (hMitChip3) and its bioinformatic tools. hMitChip3 consists of the 37 mitochondrial DNA-encoded genes, 1098 nuclear DNA-encoded and mitochondria-related genes, and 225 controls, each in triplicate. The bioinformatic tools included data analysis procedures and customized database for interpretation of results. The database associated 645 molecular functions with 946 hMitChip3 genes, 612 biological processes with 930 genes, 172 cellular components with 869 genes, 107 biological chemistry pathways with 476 genes, 23 reactome events with 227 genes, 320 genetic disorders with 237 genes, and 87 drugs targets with 55 genes. To test these tools, hMitChip3 was used to compare expression profiles between human melanoma cell lines UACC903 (rapidly dividing) and UACC903(+6) (slowly dividing). Our results demonstrated internal gene-set consistency (correlation $R \geq 0.980 \pm 0.005$) and interexperimental reproducibility ($R \geq 0.931 \pm 0.013$). Expression patterns of 16 genes, involved in DNA, RNA, or protein biosyntheses in mitochondrial and other organelles, were consistent with the proliferation rates of both cell lines, and the patterns of 6 tested genes were verified by quantitative reverse transcription PCR (RT-PCR). Thus, hMitChip3 and its bioinformatics software provide an integrated tool for profiling mitochondria-focused gene expression.

INTRODUCTION

Mitochondria, intracellular organelles widely known as the energy factories of the cell, play fundamental roles in many metabolic pathways, such as β -oxidation, the tricarboxylic acid, and urea cycles, the synthesis of steroid hormones and heme, and calcium signaling (1). Mitochondria are the only subcellular structures possessing distinct DNA (mitochondrial DNA or mtDNA) and transcription and translation machineries (2). Yet, the vast majority of mitochondrial proteins are encoded by the nuclear DNA, synthesized by ribosomes in the cytoplasm, and imported into the organelles (3). The highly integrated cross-functionality of nuclear and mitochondrial genomes is essential for maintenance of cellular homeostasis. Defects and abnormal expression of either nuclear

DNA-encoded and/or mtDNA-encoded genes can be deleterious for human organs. While nuclear DNA mutations are rare as primary mitochondrial genetic disorders (i.e., Leigh syndrome, Friedreich's ataxia, lethal infantile cardiomyopathy, carnitine palmitoyl transferase deficiency, to name a few) (4,5), abnormalities in mitochondrial structure and function are increasingly recognized in common diseases, such as obesity, diabetes, cardiomyopathy, and migraine (6–8). In addition, reactive oxygen species, an inevitable by-product of mitochondrial oxidative phosphorylation, can damage DNA and have been implicated in cancer, neurodegenerative diseases, and aging (9). Furthermore, mitochondria at the intersection of many molecular pathways are a central target of diverse pharmacological agents. Many drugs have direct effects on mitochondrial

ultrastructure and function, either at the DNA level or upon targeting proteins located in the inner or outer mitochondrial membrane (10,11). For example, curcumin and arsenic induce apoptosis via a mitochondria-mediated pathway (12,13).

A high-throughput tool for profiling transcriptomes of the entire mitochondrion would be of great importance, as it would further improve our understanding of the mitochondria-centered physiology, pathology, pharmacology, and toxicology for better diagnosis, prevention, and treatment of disease. To provide such a tool, we previously developed a first-generation human mitochondrial-focused cDNA microarray (hMitChip1, unpublished) and a second-generation human mitochondrial-focused cDNA microarray (hMitChip2) (14). hMitChip2, which contained only 501 nuclear

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DNA-encoded genes, was tested and validated in human skeletal muscle cells in an attempt to better understand mitochondrial involvement in glucocorticoid-induced myopathy (15). To make a comprehensively useful tool, we developed an integrated tool including a third-generation human mitochondria-focused cDNA microarray (hMitChip3; with 37 mtDNA-encoded genes and 1098 nuclear DNA-encoded and mitochondria-related genes), computing procedures, database, and gene informatics. We tested these tools by comparing the transcriptomes of a rapidly dividing melanoma cell line UACC903 and a slowly dividing derivative cell line UACC903(+6) (16,17). Our results demonstrate that the high quality hMitChip3 and the accompanying software provide a novel integrated tool to facilitate human mitochondria-oriented research.

MATERIALS AND METHODS

Gene Selection, Microarray Design, and Fabrication

Genes listed in the Mitoproteome database (www.mitoproteome.org/html/database.html) were selected for hMitChip3. In addition, the National Center for Biotechnology Information (NCBI) and other public databases were searched using keywords mitochondrial biogenesis and oxidative stress as previously described (14). Transcriptional loci predicted from sequences were not included. cDNA clones for the 37 human mtDNA-encoded genes were synthesized and sequence-verified by Geneart (Regensburg, Germany) based on GenBank® sequence (accession no. AP008773). Sequence-verified cDNA clones for nuclear DNA-encoded genes were purchased from Invitrogen (Carlsbad, CA, USA) and Origene (Rockville, MD, USA). Gene information was updated based on Unigene Build 189 (www.ncbi.nlm.nih.gov/UniGene). Test genes (1135), 146 positive controls (housekeeping and duplicate genes), and 79 negative controls (print-buffer without DNA) were printed in triplicate onto each hMitChip3 slide (see Supplementary

Table S1 available online at www.gwumc.edu/biochem/faculty/su.html) as previously described (18), using the OmniGrid® 100 microarrayer (Genomic Solutions, Ann Arbor,

MI, USA). Spike-in DNA controls for possible contamination (plasmid DNA), blocking efficiency (salmon sperm DNA), and cross-hybridization (human Cot-1 repetitive DNA), previ-

Figure 1. hMitChip3 representative image, quality, and data normalization. (A) Representative microarray image. This pseudocolored image represents a hMitChip3 microarray hybridized with the Cy5-labeled target cDNA reverse-transcribed from a UACC903 RNA sample. Four printing heads were used to print four subarrays of the image, and each element was printed as triplicate (with triplicates adjacent to each other). The pixel intensities on spotted probes reflect abundances of hybridized target cDNA. Inset shows four genes with high (*LOC144983* and *TUBB*), moderate (*HIP2*), and low (*MDH1*) signal intensities. (B) Bar graph illustrates mean pixel intensities of the positive ($n = 438$) and negative ($n = 237$) control spots, test gene spots ($n = 3405$), and the background of all the spots ($n = 4080$). The mean and standard deviation of the positive, test gene, negative, and background were 4456 ± 1068 , 3437 ± 602 , 464 ± 75 , and 430 ± 41 , respectively. P values (P) between comparisons are indicated. P^* means statistically significant P value ($3.62 \times 10^{-6} < 0.05$). *LOC144983*, heterogeneous nuclear ribonucleoprotein A1-like; *TUBB*, β -tubulin; *HIP2*, Huntingtin-interacting protein 2; *MDH1*, malate dehydrogenase 1. (C and D) Boxplots before and after data normalization. The quantile normalization algorithms (20) were used to adjust the natural log of the background-subtracted mean pixel intensities of each and every set of 577 genes that were selected from the hMitChip3 triplicate microarray experiments by the criteria described in the text. In contrast to (C) the prenormalization boxplots, (D) the postnormalized boxplots distribute in the same intervals with the same density center, indicating a successful location adjustment. The postnormalized data were used for further analysis to identify differentially expressed genes with statistical significance (P value < 0.05).

ously included in hMitChip2, were not necessarily repeated here (14,15).

Cell Cultures

Human melanoma cell lines UACC903 and UACC903(+6) were originally obtained from the University of Arizona Cancer Center (16). UACC903 was cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin G sodium, and 100 µg/mL streptomycin sulfate. UACC903(+6) was cultured in the same medium after addition of 600 µg/mL G418 to select for the pSV2neo-tagged chromosome 6 (16). Media, sera, and antibiotics were purchased from the GIBCO cell culture (Invitrogen). RNA samples for triplicate experiments were extracted from each cell line at the same passage cultured under the same conditions.

Microarray Hybridization and Image Scanning

Total RNA was extracted using TRIzol® reagent (Invitrogen) and purified with RNeasy® kit (Qiagen, Valencia, CA), as described previously (15). Ten micrograms RNA per sample were used for CyTM5-dUTP labeling of cDNA and microarray hybridization using methods previously described (15). Slides were scanned using the ScanArray® Express microarray scanner (PerkinElmer, Wellesley, MA, USA) under 90% laser power, 68 photomultiplier tube (PMT) voltages, 5 µm resolution, and LOWESS method. Each scanned spot was labeled either as 0 (found but not good), 1 (not found), 2 (absent), 3 (good), or 4 (bad). The good spots were defined by the scanner software setups as the spot with a calculated footprint <100 µm.

Database Compilation

A database was compiled in FileMaker Pro (FileMaker, Santa Clara, CA, USA) and includes individual and relational expression files and relational and category gene information files. Individual expression files were built for quantitative text files derived from scanned images with all

names matching exactly those of the text files. The relational expression file links all individual expression files via the index (i.e., the unique numerical identifier for each spot) and contains self-explanatory information for data filtering and comparisons. The relational gene information file contains expression summary and gene biological information. Each category gene information file organizes gene bioinformation into self-explanatory groups.

Data Analysis

Scatter plots, fitting lines, and the Pearson correlation coefficient (*R*) were calculated using Microsoft® Excel®. Microarray data were filtered within hMitChip3 database using the following criteria: (i) test gene spot = yes; (ii) spot flag = 3 (good spots); (iii) signal-to-noise ratio (i.e., spot mean pixel intensity minus background

mean pixel intensity and then divided by background standard deviation) ≥2; (iv) saturation of spot pixel intensities ≤75% (i.e., approximately linear quantitative intensities); and (v) ≥25% pixel intensities of a spot above 2 standard deviations higher than background mean intensity (i.e., good spot morphology). The background-subtracted mean intensities of the selected data were normalized across all intra- and interslide spots and array experiments. The normalized data were used to calculate average intensity, ratio, standard deviation, and *P* value.

Gene Bioinformation

Gene identifiers, including IMAGE clone ID, GenBank accession no., Unigene ID, gene ID, symbol, and name, were downloaded from the NCBI Human Unigene Database (<ftp.ncbi.nih.gov/repository/UniGene>). Gene ontology (molecular function,

Figure 2. Overview of hMitChip3 database structure. hMitChip3 database includes individual expression files, a relational expression file, a relational gene information file, and category gene information files. Each individual expression file keeps raw microarray data imported from image scanning of a microarray hybridization based on design of array experiments and is linked with the relational expression file via index (a unique numerical ID to each spot on hMitChip3) (see Figure 3A). The relational expression file contains information on array design, expression data and comparison, and some gene information (see Figure 3B). The relational gene information file contains results of expression comparisons and gene biological information (see Figure 4A) and is linked with the relational expression file via the NCBI Entrez Gene ID. All or partial information in the relational gene information file can be exported into category gene information files (see Figure 4B) for category analysis and interpretation of results. A design of microarray experiments, analysis of data, and interpretation of results need information from public databases. Arrowheads indicate the direction of information flow. Lines with dots at two ends indicate links.

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biological process, and cellular component), phenotype (genetic disorder), and pathways (KEGG pathway and reactome event) were downloaded from the Entrez Gene Database (<ftp.ncbi.nlm.nih.gov/gene>). Drug targets associated with gene products were from PharmGKB (www.pharmgkb.org/search/index.jsp).

Quantitative Reverse Transcription PCR

Two micrograms total RNA samples were reverse-transcribed into cDNA by using SuperScript™ First-Strand Synthesis System (Invitrogen). The real-time PCRs were performed using TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), following the manufacturers' instructions. Relative RNA concentrations were calculated using the published methods (15,19). TaqMan probes and primers for human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), histone deacetylase 3 (*HDAC3*), DNA (cytosine-5-)-methyltransferase 1 (*DNMT1*), chaperonin containing TCP1 (*CCT4*), nonmetastatic cell 4 (*NME4*), GA binding protein transcription factor (*GABPA*), and peroxisome proliferative activated receptor (*PPARG*) were purchased from Applied Biosystems. Triplicate quantitative reverse transcription PCR (RT-PCR) experiments were performed for each gene.

Statistics

Statistical calculations were performed on triplicate array experiments using XLSTAT 2006 (XLSTAT, New York, NY, USA). The quantile normalization described by Bolstad et al. (20) was carried out using R 2.2.1 software (The R Foundation for Statistical Computing, Vienna, Austria). As a minimum, a 2-fold difference in the background-subtracted mean intensity ratio of a gene between samples and controls was used as an indicator of differentially expressed genes. Student's *t*-tests were used to calculate *P* values for the ratio difference. The level of statistical significance was set at a *P* value < 0.05.

A. Individual Expression File			
Print Information			
Index	1	Name	HNRPA1
Array Row	1	Clone ID	27549
Array Column	1	X Dimension	3426
Spot Row	1	Y Dimension	26213
Spot Column	1		
Detection Information			
Spot Diameter	110		
Spot Pixels	348		
Background Pixels	1076.0		
Spot Footprint	13.0		
Spot Flags	3		
Statistical Information			
Spot median	14418.0		
Spot mean	13827.0		
Spot SD	7918.2		
Background median	469.0		
Background mean	515.0		
Background SD	338.8		
Percentage of Spot pixels with intensities > Background + 1 SD	96.3		
Percentage of spot pixels with intensities > B + 2 SD	96.3		
Percentage of spot pixels that are saturated	0.0		
Spot median - B	13949.0		
Spot mean - B	13358.0		
Spot signal to noise ratio (spot intensity/standard deviation of background intensity)	42.6		

B. Relational Expression File			
Links to All Data Files			
Index	1	Test Gene	Yes
		Positive C	
		Negative C	
Criteria for Filtering Data			
Cell	Chip	Flag	Signal/Noise
UACC903	L4239	3	53.0
	L4240	3	110.9
	L4241	3	58.0
	L4206	3	42.6
UACC903(+6)	L4207	3	29.7
	L4208	3	40.4
Filters*		3	≥ 2
			≤ 75
			≥ 25
* Simultaneously find test gene yes, flag 3, signal/noise ≥ 2, F% Saturation ≤ 75 and Ch1 % > B + 2 SD ≥ 25			
Normalized Mean-B			
Cell	Chip	Set 1	Set 2
UACC903	L4239	9559	1191
	L4240	1724	1362
	L4241	7648	7933
	L4206	1336	1362
UACC903(+6)	L4207	1300	1566
	L4208	1032	1111
			9855
			2215
			7584
			1592
			1392
			9954

Figure 3. User interface-1 of hMitChip3 database compiled in FileMaker Pro. (A) Individual expression file allows storage and display of the print information, detection information, and statistical information imported directly from output of scanned Excel text file. All names listed are the same as those of the text file. (B) Relational expression file links all individual expression files via the index (i.e., the unique numerical identifier for each spot) and contains self-explanatory information for data filtering and comparisons. The databases allow searching, browsing, displaying, and modifying, according to experimental designs. Templates of hMitChip3 database with self-explanatory information can be downloaded at www.gwumc.edu/biochem/faculty/su.html.

RESULTS

Uniformly Low Background of the hMitChip3 Gene Chips

The quality of hMitChip3 slides was assessed in triplicate microarray experiments using RNA samples derived from human melanoma cell lines UACC903 and UACC903(+6). Results showed uniformly low background intensity on all hybridized chips, with a distribution of signal intensities from 65,535 pixels (<1% of 4080 spots) gradually down to the background (Figure 1A). Specifically, the averages of the mean pixel intensities for the positive, test, background, and negative spots (see Supplementary Table S1), calculated from all the six hybridized chips (regardless of the RNA sample used), were 4456 ± 1067 , 3437 ± 602 , 430 ± 41 , and 464 ± 75 , respectively (Figure 1B). On average, the signal intensities of the test spots (3437 pixels) were 8-fold higher than those of the background (430 pixels), and this difference was highly significant ($P = 3.62 \times 10^{-6}$). In contrast, the difference between test and positive spots was not statistically significant ($P = 0.069$).

There was essentially no difference in mean intensities between negative spots and background ($P = 0.337$) (Figure 1B).

High Signal-to-Noise Ratio and Equal Overall Spot Pixel Intensities

Table 1 lists the signal-to-noise ratios and averages of the background-subtracted mean pixel intensities. On average, the signal-to-noise ratio was 12.6-fold for the test gene spots and 16.6- and 1.4-fold for the positive and the negative controls, respectively. The corresponding spot background-subtracted mean pixel intensities were 3064, 4079, and 93, respectively. The difference in average signal-to-noise ratios between UACC903 and UACC903(+6) cDNA hybridized chips were 1.27, 1.42, and 0.82 for test genes, positive, and negative controls, respectively. The corresponding difference in the spot background-subtracted mean pixel intensities between the two cell lines was 1.30, 1.38, and 1.07, respectively. These differences were not statistically significant (Table 1), although the average signal-to-noise ratios and spot background-subtracted mean pixel

intensities of the test genes and positive controls for UACC903 were slightly higher than those for UACC903(+6). Therefore, high signal-to-noise ratio and equal overall pixel intensities for UACC903 and UACC903(+6) samples were obtained using hMitChip3.

Similarity Between the Identical Gene-Set Intensities

To compare intensities among identical gene-sets measured by hMitChip3, we conducted 2×2 comparisons of the spot background-subtracted mean pixel intensity for the test genes and positive controls on six hybridized chips and calculated Pearson correlation coefficients (R) for the Proximity Matrix (Table 2). The matrix showed intensity similarities (R) for each set of genes, both within the same chip and between different chips, as well as between RNA samples obtained from the two different cell lines. The intra-hMitChip3 values of the mean similarity and standard deviation were 0.980 ± 0.005 and 0.981 ± 0.011 for UACC903 and UACC903(+6), respectively. The corresponding inter-hMitChip3 values were 0.931 ± 0.013 and 0.944 ± 0.017 , respectively. The mean similarity and standard deviation between UACC903 and UACC903(+6) samples were 0.879 ± 0.020 , which as expected was the least among all the correlation coefficients (Table 2).

Computing Procedures for Identification of Differentially Expressed Genes

To identify differentially expressed genes, we customized computational procedures including data evaluation, filtering, inclusion, normalization, and comparison. Data evaluation is aimed at revealing data quality including background, signal-to-noise ratio, overall spot pixel intensity, and consistency. Data filtering is aimed at removing (with uniform criteria described in the Materials and Methods section) all spots that may lead to false positives or negatives. Data inclusion is aimed at determining whether all spots fulfilling the criteria for data filtering should be used for

further analysis. Because some of the 9 spots/gene (i.e., triplicate spots/chip from triplicate experiments) may not fulfill the filtering criteria, some of the filtered genes across all array experiments may have values from <9 spots. Alternatively, one may include only genes with all the 9 spots fulfilling the filtering criteria for further analysis. The former complicates data analysis because of missing values. The latter gave a subset of genes with no missing data; however, the number of genes included in the final result may be reduced. In the current experiments, 2029 of 3405 test gene spots satisfied the filtering criteria. Of these 2029 spots, 1731 (85%) represented the 577 genes for which all the 18 spots/gene (i.e., triplicate spots/chip, from triplicate array experiments performed in two cell lines) met the criteria; 240 (12%) spots represented the 120 genes for which 12 out of 18 spots/gene and 6 spots/cell line passed the filtering criteria; and 58 (3%) spots represented the 58 genes with 6 spots/gene and 3 spots/cell line that passed the filtering criteria. In this analysis, only the 577 genes were used for further analysis.

Data normalization is aimed at removing nonbiological variations that can arise in any steps of microarray experiments, including array printing, RNA preparation, labeling, hybridization, or scanning (21). Before and after performing quantile normalization (20), the background-subtracted mean pixel intensities of 10,386 spots (i.e., triplicate spots/gene/chip, of 577 genes included, from triplicate array experiments, performed on two cell lines) were transformed to natural log. The log-transformed values were plotted as the boxplots to visualize successful adjustment of the data by graphical comparisons (Figure 1, C and D).

Data comparison is to compute averages, standard deviations, ratios, and P values of the normalized spot background-subtracted mean pixel intensities between desired comparisons in order to reveal differentially expressed genes. An intra- and inter-hMitChip3 average of triplicate gene-sets and triplicate experiments should reduce variance in pixel intensities. Ratios of averages between test and reference samples provide comparisons

of transcriptional profiles. P values are calculated from triplicate array experiments ($n = 3$) (i.e., 3 averages of triplicate gene-sets/array experiment for each cell line). When using 2-fold difference as threshold, we identified 66 differentially expressed genes between UACC903 and UACC903(+6) cells (Supplementary Table S2).

hMitChip3 Database

To expedite data analysis and interpretation of results, we built the hMitChip3 database for data organization, computation, interpretation, and visualization. Figure 2 is a diagrammatical overview of the hMitChip3 database. The individual expression files are used to store microarray data, including printing, detection, and statistical information (Figure 3A). The relational expression file links all the individual expression files and provides user-interface to allow importing, searching, browsing, computing, comparing, displaying, and exporting of data (Figure 3B). Containing gene bioinformation, the relational gene information file (Figure 4A) provides user-interface to allow importing, searching, browsing, displaying, and exporting of (i) genes of interest, with the associated expression changes and bioinformation; (ii) expression changes of interest, with the associated genes and bioinformation; and (iii) bioinformation of interest, with the associated genes and expression results. The category gene information files consist of individual files of molecular function, biological process, cellular component, KEGG pathway, reactome event, genetic disorder, and PharmGKB drug targets. Because of multiple genes under each bioinformation and multiple bioinformation under each gene, and their association with expression changes, information in each category file can be organized by sorting based on genes, expression, or bioinformation of interest. As an example, Figure 4B shows partial biological process focused on anti-apoptosis. Both relational and category gene information files are linked to public databases via the NCBI Entrez Gene ID. Templates of hMitChip3 database with self-explanatory infor-

mation can be downloaded at www.gwumc.edu/biochem/faculty/su.html.

hMitChip3 Gene Bioinformation

Bioinformation of 1135 hMitChip3 test genes were compiled and imported into relational and category gene information files to facilitate the biological interpretation of differentially expressed genes. Specifically, we were able to link 946 hMitChip3 genes with 645 molecular functions (Supplementary Table S3), 930 genes with 612 biological processes (Supplementary Table S4), 869 genes with 172 cellular components (Supplementary Table S5), 476 genes with 107 KEGG biological chemistry pathways (Supplementary Table S6), 227 genes with 23 reactome events (Supplementary Table S7), 237 genes with 320 genetic disorders (Supplementary Table S8), and 55 genes with 87 PharmGKB drug targets (Supplementary Table S9). All supplementary tables are available online at www.gwumc.edu/biochem/faculty/su.html.

Quantitative RT-PCR Verification of Differentially Expressed Genes

Applying the hMitChip3 gene information to 66 differentially expressed genes (Supplementary Table 2), we found 16 genes with known functions involved in cell cycle progression and DNA, RNA, or protein biosynthesis (Table 3) for mitochondrial and other organelles. The reason focusing on these functions is that a growth rate of UACC903 cell line is twice as rapid as that of UACC903(+6) cell line (17). Our quantitative RT-PCR results on six tested genes demonstrated agreement with the microarray results (Table 3). Specifically, the complete consistent expression changes include *HDAC3* (2.96-fold, *P* = 0.0386), *DNMT1* (1.69-fold, *P* = 0.0287), and *NME4* (1.64-fold, *P* = 0.0327) with higher expression and *PPARG* (0.18-fold, *P* = 0.00000004) with lower expression in UACC903 than in UACC903(+6). *CCT4* and *GABPA* were up-regulated by 1.81-fold (*P* = 0.2638) and 2.03-fold (*P* = 0.2626) higher, respectively, in UACC903 than UACC903(+6);

Table 1. S/N Ratio and Average Intensitives of hMitChip3 Spots

RNA	Chip	Spot		Average		UACC903 vs. UACC903(+6)			
		Group	<i>n</i>	S/N	S-B Mean	S/N	<i>P</i> Value	S-B Mean	<i>P</i> Value
UACC903	L4239	Test	3405	12.5	3076				
		Positive	438	14.5	3653				
		Negative	237	1.2	104				
	L4240	Test	3405	15.3	3756				
		Positive	438	23.4	5455				
		Negative	237	1.2	75				
	L4241	Test	3405	14.4	3577				
		Positive	438	20.5	5064				
		Negative	237	1.5	109				
UACC903(+6)	L4206	Test	3405	11.5	2856				
		Positive	438	14.3	3757				
		Negative	237	1.5	50				
	L4207	Test	3405	9.3	2064				
		Positive	438	9.5	2430				
		Negative	237	1.3	40				
	L4208	Test	3405	12.5	3057				
		Positive	438	17.2	4115				
		Negative	237	2.0	179				
Test				12.6	3064	1.27	0.08	1.30	0.09
Positive				16.6	4079	1.42	0.17	1.38	0.16
Negative				1.4	93	0.82	0.29	1.07	0.89
S/N, signal-to-noise ratio; S-B Mean, spot background-subtracted mean pixel intensities.									

S/N, signal-to-noise ratio; S-B Mean, spot background-subtracted mean pixel intensities.

however, their *P* values, in contrast to microarray results, were >0.05.

DISCUSSION

Gene expression profiling by DNA microarrays allows high-throughput screening for genes and pathways with key functions in diverse physiologic, pathologic, pharmacologic, and toxicologic processes. Mitochondrial research has influenced evolving concepts in evolution, physiology, pathology, pharmacology, and toxicology. An increasingly better understanding of mitochondrial disorders and the association of mitochondrial impairment with a constantly expanding list of common diseases have attracted the attention of the scientific and medical communities (1,22). Accordingly, content in the human mitochondrial genome database MITOMAP has grown rapidly (23). A Human Mitochip was developed

for a high-throughput screening of mutations in the mitochondrial genome (24). In addition, a cDNA microarray containing 618 genes was used to study human mitochondrial complex 1 deficiency (25), and generic cDNA microarrays and oligonucleotide gene chips have also been utilized to study mitochondrial dysfunction (26).

We previously developed and utilized hMitChip2 to study the mitochondrial involvement in glucocorticoid-induced myopathy (15,27). Because hMitChip2 contained no mtDNA-encoded genes and only 501 nuclear DNA-encoded genes (14), we improved the microarray by developing hMitChip3. To our knowledge, hMitChip3 is the first customized human cDNA microarray containing all the 37 mtDNA-encoded genes and 1098 nuclear DNA-encoded and mitochondria-related genes, allowing intergenomic studies of the mitochondrial genes. The advantages

of a focused microarray over human genome microarray with estimated 30,000 genes include hypothesis-driven experiments, affordable costs, simplified data analysis, and defined biological interpretation of results. However, considering that the sequence-predicted DNA loci were not included in hMitChip3 and that the number of the estimated mitochondrial genes (26) and proteins (28) are constantly increasing, a disadvantage of hMitChip3 in contrast to the whole genome gene chip probably is absence of genes that might be important to mitochondrial function.

We achieved high quality and consistency for hMitChip3 by using the highest standards in the choice of manufacturing sources and materials, design, development, and quality control. The test, positive, and negative controls were printed in triplicate on each slide to reduce random variance. For both positive controls and test genes, >13-fold signal-to-noise ratio was consistently reached when using 10- μ g RNA for labeling and hybridization, whereas the ratios of the negative controls were, as expected, around 1. The uniformly low background (<500 pixel intensities) did not change when using 50- μ g total RNA, and in this case, the average signal-to-noise ratio ranged as high as 70-fold (14). Although using more RNA will increase the signal-to-noise ratio and lead to detection of rare RNA species, it also increased signal

saturation of abundant RNA species (14). Therefore, one should adjust the amount of RNA based on an experimental design. Importantly, the equal amount of RNA samples labeled and hybridized to the spotted DNA probes on each hMitChip3 were authentically translated into the overall equal spot pixel intensities. The overall equality is essential to the identification of differentially expressed individual genes.

Nonbiological variations interfere with true biological variations and may cause false positives and negatives of differentially expressed genes. Nonbiological variations can arise in any step, including printing arrays, RNA sample preparation, labeling, hybridization, or image scanning (21). Technical variations can be removed by filters (29,30). For example, spots below a detection threshold for the amount of RNA used ought to be removed for obvious reasons. In addition, spots with low intensities, lack of linear quantitation, or irregular morphology should also be excluded for lack of reliability. The hMitChip3 database simplifies the data filtering procedures into a single step: that is, to simultaneously find the flag 3, the signal-to-noise ratio ≥ 2 , the percentage of spot saturation $\leq 75\%$, and the spot pixel intensity >2 standard deviations of background ≥ 25 , across all test genes in all array experiments.

Nonbiological variations may be mixed with meaningful biological signals in the selected spots and

require more sophisticated methods to decipher. To date, a number of algorithms to normalize data from various array platforms have been developed to accomplish such a daunting task (20,31–34). Because there is no gold standard to identify a best normalization method for any array platform, we decided to utilize the quantile algorithm to normalize the hMitChip3 data, partially because of the single-fluorescent (Cy5) labeling and partially because of the simplicity of this algorithm (20). It should be noted that while the positive and negative controls on hMitChip3 provide good indicators for high, medium, low, and background intensities, they are not immune to nonbiological variations.

DNA microarrays have used either one- or two-fluorescent approaches to quantify RNA in cell and tissue samples. The single-fluorescent approach is to synthesize Cy3- or Cy5-labeled cDNAs for all RNA samples, hybridize to arrayed probes, measure the background-subtracted intensity at each probe on microarrays, and calculate the ratio of gene expression. The two-fluorescent approach is to synthesize Cy3- and Cy5-labeled test and reference cDNA samples individually and co-hybridize them to arrayed probes, and directly measure the ratio of the background-subtracted intensities at each probe. The pros and cons of both approaches are well-known (35,36). For the single-

A. Relational Gene Information File						
Gene						
ID	8841	Web	Symbol	HDAC3		
Name	histone deacetylase 3					
Expression						
Sample	N	Mean	SD	Comparison	Ratio	Log2 P
UACC903	3	4528	568	UACC903 vs 9036	6.65	2.73 0.0004
UACC903(+6)	3	681	263			
NCBI Entrez Gene Summary						
<p>"Histones play a critical role in transcriptional regulation, cell cycle progression, and developmental events. Histone acetylation/deacetylation alters chromosome structure and affects transcription factor access to DNA. The protein encoded by this gene belongs to the histone deacetylase/acetylase family. It has histone deacetylase activity and represses transcription when tethered to a promoter. It may participate in the regulation of transcription through its binding with the zinc-finger transcription factor YY1. This protein can also down-regulate p53 function and thus modulate cell growth and apoptosis. This gene is regarded as a potential tumor suppressor gene."</p>						
NCBI Entrez and PharmGKB Gene Information						
Molecular Function	histone deacetylase activity					
Biological Process	regulation of progression through cell cycle; anti-apoptosis					
Cellular Component	cytoplasm					
Description	ID#					
KEGG Pathway						
Reactome Event						
Genetic Disorder						
PharmGKB Drug						
PubMed Refs.	the inhibition of p15(NK4b) and p21(WAF1/cip1) may be one of the mechanisms by which HDAC3 participates in cell cycle regulation and oncogenesis.					16298343

B. Category Gene Information File			
Biological Process			
Gene ID	Symbol	Name	
anterograde axon cargo transport			
23095	KIF1B	kinesin family member 1B	
anti-apoptosis			
301	ANKK1	ankyrin A1	
306	ANKK5	ankyrin A5	
8539	AP5	apoptosis inhibitor 5	
596	BCL2	B-cell CLL/lymphoma 2	
597	BCL2A1	BCL2-related protein A1	
598	BCL2L1	BCL2-like 1	
19017	BCL2L10	BCL2-like 10 (leukostasis facilitator)	
662	BNP1	BCL2adenovirus E1B 19kDa interacting protein 1	
664	BNP5	BCL2adenovirus E1B 19kDa interacting protein 3	
673	BRAF	v-rat murine sarcoma viral oncoprotein homolog B1	
835	CASP2	caspase 2, apoptosis-related cysteine protease (neuronal precursor cell)	
355	FAS	Fas (TNF receptor superfamily member 6)	
8841	HDAC3	histone deacetylase 3	
3309	HSPA5	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	
3313	HSPA9B	heat shock 70kDa protein 9B (montain-2)	
4170	MLL1	myeloid cell leukemia sequence 1 (BCL2-related)	
4353	MPO	myeloperoxidase	
7001	PRD3	perlecanin 2	
5653	PSDN1	presenilin 1 (Alzheimer disease 3)	
7428	VHL	von Hippel-Lindau tumor suppressor	
antimicrobial humoral response (sensu Vertebrata)			
1536	CYBB	cytochrome b-245, beta subunit (chronic granulomatous disease)	
8772	FADD	Fas (TNFRSF6)-associated via death domain	
4259	MGST3	mitochondrial glutathione S-transferase 3	
apical junction assembly			
1455	CTNNA1	catenin (cadherin-associated protein), alpha 1, 102kDa	
apoptosis			
8539	AP5	apoptosis inhibitor 5	
351	APP	amyloid beta (A4) precursor protein (cystic disease, Alzheimer disease)	
581	BAX	BCL2-associated X protein	
27113	BBC3	BCL2 binding component 3	
10134	BCAP31	B-cell receptor-associated protein 31	
662	BNP1	BCL2adenovirus E1B 19kDa interacting protein 1	

Figure 4. User interface-2 of hMitChip3 database compiled in FileMaker Pro. (A) Relational gene information file contains gene expression summary and biological information as listed. All gene IDs are hyperlinked to the NCBI Entrez Gene web site for instant checking or updating information. (B) An example of category gene information file—Biological Process. Seven category gene information files include molecular function, biological process, cellular component, KEGG pathway, reactome event, genetic disorder, and PharmGKB drug targets. Each category gene information file is to organize gene bioinformation into self-explanatory functional clusters to facilitate interpretation of gene expression profile results. The databases allow searching, browsing, displaying, modifying, and updating information. Templates of hMitChip3 database with self-explanatory information can be downloaded at www.gwumc.edu/biochem/faculty/su.html.

Table 2. Proximity Matrix (Pearson Correlation Coefficient, *R*) of RNA Levels Measured by hMitChip3

RNA			UACC903(+6)									UACC903								
hMitChip3			L4206			L4207			L4208			L4239			L4240			L4241		
Genes			Set 1	Set 2	Set 3	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
UACC903(+6)	Set 1	1	0.982	0.969	0.969	0.950	0.919	0.942	0.966	0.969	0.969	0.859	0.864	0.871	0.880	0.899	0.906	0.875	0.900	0.904
	Set 2	0.982	1	0.963	0.955	0.925	0.947	0.960	0.960	0.964	0.965	0.861	0.866	0.873	0.876	0.902	0.911	0.858	0.890	0.893
	Set 3	0.969	0.963	1	0.936	0.908	0.929	0.951	0.951	0.954	0.955	0.847	0.853	0.861	0.871	0.890	0.897	0.854	0.880	0.883
	Set 1	0.950	0.955	0.936	1	0.978	0.989	0.945	0.945	0.952	0.950	0.869	0.887	0.895	0.854	0.894	0.897	0.846	0.892	0.890
	Set 2	0.919	0.925	0.908	0.978	1	0.972	0.916	0.916	0.923	0.921	0.840	0.862	0.867	0.822	0.865	0.868	0.815	0.865	0.861
	Set 3	0.942	0.947	0.929	0.989	0.972	1	0.935	0.935	0.942	0.941	0.861	0.882	0.888	0.844	0.887	0.889	0.834	0.884	0.881
	Set 1	0.966	0.960	0.951	0.945	0.916	0.935	1	0.993	0.993	0.994	0.883	0.883	0.895	0.881	0.895	0.901	0.872	0.894	0.888
	Set 2	0.969	0.964	0.954	0.952	0.923	0.942	0.993	1	0.993	0.993	0.883	0.885	0.894	0.878	0.899	0.903	0.866	0.893	0.888
	Set 3	0.969	0.965	0.955	0.950	0.921	0.941	0.994	0.994	0.993	1	0.891	0.891	0.902	0.890	0.906	0.911	0.878	0.901	0.897
	Set 1	0.859	0.861	0.847	0.869	0.840	0.861	0.883	0.883	0.883	0.891	1	0.984	0.983	0.931	0.935	0.934	0.923	0.938	0.921
UACC903	Set 2	0.864	0.866	0.853	0.887	0.862	0.882	0.883	0.883	0.885	0.891	0.984	1	0.988	0.907	0.927	0.925	0.904	0.931	0.917
	Set 3	0.871	0.873	0.861	0.895	0.867	0.888	0.895	0.895	0.894	0.902	0.983	0.988	1	0.916	0.932	0.931	0.912	0.936	0.924
	Set 1	0.880	0.876	0.871	0.854	0.822	0.844	0.881	0.881	0.878	0.890	0.931	0.907	0.916	1	0.973	0.973	0.952	0.947	0.939
UACC903	Set 2	0.899	0.902	0.890	0.894	0.865	0.887	0.895	0.895	0.899	0.906	0.935	0.927	0.932	0.973	1	0.983	0.935	0.952	0.945
	Set 3	0.906	0.911	0.897	0.897	0.868	0.889	0.901	0.901	0.903	0.911	0.934	0.925	0.931	0.973	0.983	1	0.935	0.950	0.944
	Set 1	0.875	0.858	0.854	0.846	0.815	0.834	0.872	0.872	0.866	0.878	0.923	0.904	0.912	0.952	0.935	0.935	1	0.980	0.975
UACC903	Set 2	0.900	0.890	0.880	0.892	0.865	0.884	0.894	0.894	0.893	0.901	0.938	0.931	0.936	0.947	0.952	0.950	0.980	1	0.984
	Set 3	0.904	0.893	0.883	0.890	0.861	0.881	0.888	0.888	0.888	0.897	0.921	0.917	0.924	0.939	0.945	0.944	0.975	0.984	1
	When measured by the gene-sets on the same hMitChip3 (gray), the mean and standard deviation (sd) of Pearson correlation coefficient (<i>R</i>) of spot intensities between the UACC903(+6) RNA samples were 0.981 ± 0.011, and they were 0.980 ± 0.005 between UACC903. When measured by hMitChip3 in triplicate array experiments (italicized), the mean and sd of <i>R</i> between the UACC903(+6) RNA samples were 0.944 ± 0.017, and they were 0.931 ± 0.013 between UACC903. The mean and sd of <i>R</i> between the UACC903 and UACC903(+6) RNA samples (underlined) were 0.879 ± 0.020.																			

fluorescent approach, some studies showed evidence for better gene classifiers (37) or accurate expression ratios (38). hMitChip3 as a cDNA microarray can be used with either the one- or two-fluorescent approach according to users' preferences. In this study, the hMitChip3 slides were hybridized with single dye (Cy5)-labeled target DNA from both the test and the reference samples, and the results were validated by quantitative RT-PCR. Two dyes, Cy3- and Cy5-labeling, were tested and validated in experiments conducted with hMitChip2 (14) and remains applicable to hMitChip3.

Microarray data frequently contain a large amount of information on thousands of genes requiring sophisticated bioinformatics tools for appropriate data organization, analysis, and interpretation. The hMitChip3 database, computing procedure, and gene bioinformatics reported herein provide such the tools for rapid identification of

candidate genes and biological interpretation of results. The hMitChip3 database structure can be readily modified not only for hMitChip3-derived data but also for data generated from other gene chips. The linking of hMitChip3 database to public web sites allows investigators to instantaneously update gene information. Because the output of the array scan is a text file, experienced microarray investigators might select a variety of analytical tools for further data analysis.

The number of candidate genes detected by hMitChip3 is small enough to eliminate the need for high-order analyses such as hierarchical clustering and multidimensional scaling that are based on similarity in gene expression. Instead, a joint mining of differentially expressed genes and biological information within the hMitChip3 database allowed focusing on biological functions relevant to experimental design and interpretation of results. Out of 66 differen-

tially expressed genes mapped to specific biological functions, 6 mtDNA-encoded genes and 10 nuclear DNA-encoded genes have known functions involved in cell cycle progression and DNA, RNA or protein biosynthesis, and displayed expression patterns consistent with rapid (UACC903) or slow [UACC903(+6)] proliferation status of the cell lines. Although the quantitative RT-PCR verified expression patterns of 6 tested genes, differences indeed exist between microarray and quantitative RT-PCR. In this regard, the differential expression of *CCT4* and *GABPA* detected by quantitative RT-PCR were statistically not significant ($P > 0.05$), in contrast to microarray results. The difference might be partially due to computational difference (577 selected genes used for microarray data normalization versus a single gene *GAPDH* for quantitative RT-PCR) and partially due to technical difference (higher sensitivity of quantitative RT-PCR than that of microarray).

Table 3. Genes Differentially Expressed between UACC903 and UACC903(+6) Cell Lines

Symbol	Name	Activity	Microarray		Quantitative RT-PCR	
			903/903(+6)	P	903/903(+6)	P
1. Cell Cycle Progression						
HDAC3	histone deacetylase 3	positive effect	6.65	0.000	2.96	0.039
DNMT1	DNA (cytosine-5-)-methyltransferase 1	positive effect	4.36	0.000	1.69	0.029
CCT4	chaperonin containing TCP1 subunit 4	positive effect	2.06	0.021	1.81	0.264
2. Nucleotide Synthesis						
NME4	nonmetastatic cells 4, protein expressed in	[d]NTP synthesis	5.66	0.014	1.64	0.033
3. RNA						
GABPA	GA binding protein transcription factor α	transcription factor	4.07	0.001	2.03	0.263
PPARG	peroxisome proliferative activated receptor γ	transcription factor	0.27	0.002	0.18	0.000
POLR3E	polymerase (RNA) III (DNA directed) polypeptide E	tRNA, 5S rRNA, U6 snRNA synthesis	2.73	0.007	ND	
HNRPD	heterogeneous nuclear ribonucleoprotein D	RNA stability	2.62	0.006	ND	
4. Protein						
HARS	histidyl-tRNA synthetase	histidine activation	3.56	0.002	ND	
SARS2	seryl-tRNA synthetase 2	serine activation	2.33	0.011	ND	
5. Mitochondria						
MTIF3	mitochondrial translational initiation factor 3	translation initiation	3.53	0.001	ND	
TUFM	Tu translation elongation factor, mitochondrial	translation elongation	3.78	0.000	ND	
MRPS16	mitochondrial ribosomal protein S16	translation	2.04	0.027	ND	
MRPS23	mitochondrial ribosomal protein S23	translation	5.27	0.000	ND	
TRNE	tRNA glutamic acid	glutamic acid carrier	3.88	0.005	ND	
TRNY	tRNA tyrosine	tyrosine carrier	2.96	0.034	ND	
RT-PCR, reverse transcription PCR; TCP1, T-complex protein 1; tRNA, transfer RNA; rRNA, ribosomal RNA; snRNA, small nuclear RNA; ND, not determined.						

RT-PCR, reverse transcription PCR; TCP1, T-complex protein 1; tRNA, transfer RNA; rRNA, ribosomal RNA; snRNA, small nuclear RNA; ND, not determined.

Therefore, we recommend validation of microarray-detected candidate genes by the different method prior to functional study.

Genes involved in cell proliferation were, as expected, up-regulated in a rapidly dividing melanoma cell line (UACC903) in contrast to a slowly dividing derivative cell line [UACC903(+6)]. *HDAC3* is the main component of the nucleosome remodeling and deacetylase complex that represses transcription of specific genes with negative control on cell cycle to enhance cell proliferation (39). *DNMT1* binds to histone deacetylases and retinoblastoma (Rb) tumor suppressor, leading to loss of functional Rb (40). *CCT4* is required for maturation of cyclin E and the cell cycle progression from G1 to S phase (41). *NME4* is a nucleoside diphosphate kinase that is involved in RNA or DNA biosynthesis (42). *GABPA* is the α subunit of GA binding protein that regulates genes involved in cell cycle control, protein biosynthesis, and cellular metabolism (43). In contrast, expression of *PPARG*, a differentiation-related transcription factor (44), was down-regulated in UACC903 cells compared with UACC903(+6) cells. Genes involved in protein biosynthesis were up-regulated in UACC903 in contrast to UACC903(+6) and have yet to be validated. Taken together, the results in this report validate hMitChip3 slides as a measurement tool and hMitChip3 database and computing procedures as an analytic tool, useful to profiling mitochondrial gene expression.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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