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Genomic Profiling of Neutrophil Transcripts in Asian *Qigong* Practitioners: A Pilot Study in Gene Regulation by Mind–Body Interaction

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ABSTRACT

Background and objectives: The great similarity of the genomes of humans and other species stimulated us to search for genes regulated by elements associated with human uniqueness, such as the mind–body interaction. DNA microarray technology offers the advantage of analyzing thousands of genes simultaneously, with the potential to determine healthy phenotypic changes in gene expression. The aim of this study was to determine the genomic profile and function of neutrophils in Falun Gong (FLG, an ancient Chinese *Qigong*) practitioners, with healthy subjects as controls.

Subjects and design: Six (6) Asian FLG practitioners and 6 Asian normal healthy controls were recruited for our study. The practitioners have practiced FLG for at least 1 year (range, 1–5 years). The practice includes daily reading of FLG books and daily practice of exercises lasting 1–2 hours. Selected normal healthy controls did not perform *Qigong*, yoga, t'ai chi, or any other type of mind–body practice, and had not followed any conventional physical exercise program for at least 1 year. Neutrophils were isolated from fresh blood and assayed for gene expression, using microarrays and RNase protection assay (RPA), as well as for function (phagocytosis) and survival (apoptosis).

Results: The changes in gene expression of FLG practitioners in contrast to normal healthy controls were characterized by enhanced immunity, downregulation of cellular metabolism, and alteration of apoptotic genes in favor of a rapid resolution of inflammation. The lifespan of normal neutrophils was prolonged, while the inflammatory neutrophils displayed accelerated cell death in FLG practitioners as determined by enzyme-linked immunosorbent assay. Correlating with enhanced immunity reflected by microarray data, neutrophil phagocytosis was significantly increased in *Qigong* practitioners. Some of the altered genes observed by microarray were confirmed by RPA.

Conclusion: *Qigong* practice may regulate immunity, metabolic rate, and cell death, possibly at the transcriptional level. Our pilot study provides the first evidence that *Qigong* practice may exert transcriptional regulation at a genomic level. New approaches are needed to study how genes are regulated by elements associated with human uniqueness, such as consciousness, cognition, and spirituality.

INTRODUCTION

There has been increasing interest in the phenomenon of the mind–body interaction, a subject that had been considered difficult to study due to its complex nature. However, there is ample evidence to support the powerful physical effects of practices that are directed toward the mind or spirituality. While the health benefits of yoga, meditation, and prayer have been documented and well recognized by the general public (Cha et al., 2001; Gimbel, 1998; Krucoff

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et al., 2001; Pettinati, 2001; Sovik, 2000), the mechanisms underlying such apparent effects are not well understood at the physiologic level and, in particular, have not been documented and systematically studied at the cellular or molecular level.

The genomes of all mammals are so similar that it is difficult to understand how similar genetic material can generate so many different living creatures (Paabo, 2001). Recently it was confirmed that mice and humans share more than 97% of their genetic material, and one of the differences between the mouse and human genomes lies in the activity of junk DNA sequences that do not code for proteins (Dennis, 2002; Waterston et al., 2002). What "magic" factors could possibly be beyond the one-dimensional genome? If genes of different animals are similar, then it is possible that other factors, such as changes in gene regulation mediated by the mind–body interaction, might drive the differences between humans and other species. A great body of knowledge regarding gene regulation by a variety of physical and pathologic insults has been obtained; however, little has been done regarding how mind–body interaction regulates gene expression, what mind-associated genes are, and how mind-associated genes relate to or control the genes specifically regulated by physical and pathologic stimuli.

A spiritual perspective in medical practice and research has been advocated with increasing urgency in mainstream medical journals during the past few years (Chandler, 1999; Emanuel et al., 1998; Libert, 2003; Post et al., 2000; Tracey, 2002). In ancient China there were numerous practices to help improve health by simultaneously reinforcing body, mind, and spirit. Many of these practices we now call *Qigong* (pronounced chi-kung) are recognized today as transitional health practices and healing techniques. Falun Gong (FLG), also called Falun Dafa, is one type of these ancient Chinese *Qigong* practices, and consists of exercise–meditation to energize the physical body, along with an emphasis on the spiritual practice, based on the triad of Truthfulness, Compassion, and Forbearance. It has been reported that FLG exhibits very dramatic and powerful effects on practitioners (Dan et al., 1998). Although spiritual practice has been thought to have a great impact on the human body, how the mind could be specifically involved in regulation of gene expression remains to be determined at both the molecular and cellular levels.

DNA microarrays are powerful tools to monitor gene expression in both illness and health. The advantage of microarray analyses includes the ability to study the regulation of several genes or even the entire genome in a single experiment. In this study, we examined the genomic profile as well as functional alteration of neutrophils from FLG practitioners. Our studies indicate modern technology may be used as a scientific tool to study the molecular mechanism of health benefits seen in people practicing spirituality or employing complementary and alternative medicine.

MATERIALS AND METHODS

Subjects

Eligible subjects were 18 years of age or older. Six (6) Asian FLG practitioners (3 males and 3 females, mean age, 46.7 ± 13.3 years) and 6 Asian normal healthy controls (3 males and 3 females, mean age, 41.3 ± 11.3 years) were recruited by advertisement for our study. The practitioners had practiced FLG for at least 1 year (range, 1–5 years). The practice includes daily book reading (Li, 1994) and daily FLG exercises lasting 1–2 hours each time. Selected normal healthy controls had not performed *Qigong*, yoga, *t'ai chi*, any type of mind–body practice, or physical exercise for at least 1 year. With the approval of the Institutional Review Board and after informed consent was given, 30 mL of heparinized blood was taken from each subject.

Cell preparation

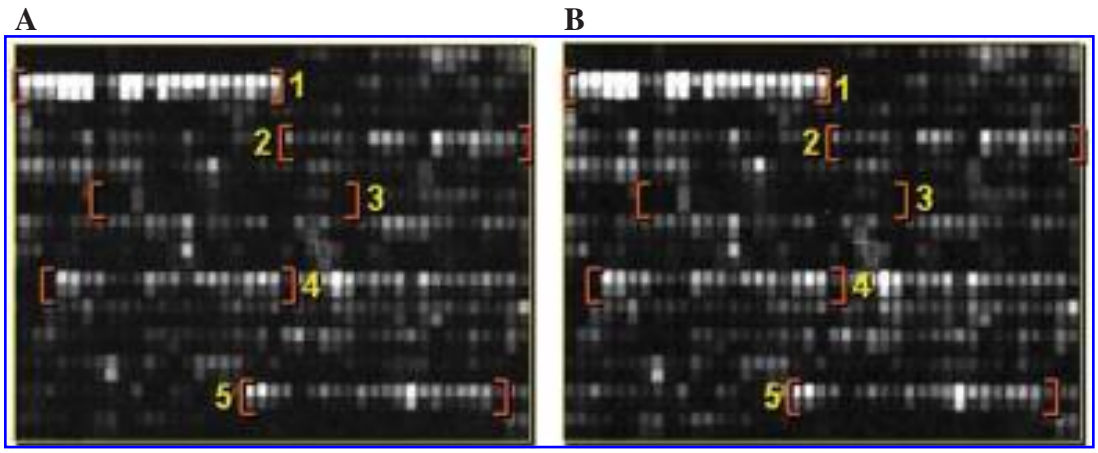
Venous blood samples were collected into heparinized syringes, and cells were separated immediately. Polymorphonuclear leukocytes (PMN) were prepared from heparinized blood by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation, followed by dextran sedimentation. Contaminating erythrocytes were not removed, to avoid further damage. Neutrophils were subjected to further experiments without hypotonic treatment, since it was found that hypotonic treatment might damage neutrophils. Freshly isolated human neutrophils were resuspended in phosphate buffered saline (PBS) and enumerated by Coulter analyzer (Coulter Corporation, Miami, FL). Microscopic examinations revealed that 96–98% of the cells were neutrophils and that more than 95% of the cells were viable by a trypan blue exclusion test.

Cell culture and lipopolysaccharide (LPS) stimulation

Neutrophils were finally resuspended in RPMI 1640 with 10% fetal bovine serum (FBS) and adjusted to appropriate concentration. The purity of PMN isolated was always $>98\%$, as determined by Giemsa staining. PMN preparations consistently contained $<1\%$ monocytes. The PMN viability was $>98\%$, as estimated by trypan blue exclusion. When cultured in the presence or absence of lipopolysaccharide (LPS), neutrophils were harvested at 0, 4, 8, 16, and 24 hours for further analysis.

Light microscopy

The morphologic changes of apoptosis were demonstrated also by light microscopy examination of Wright-stained cytospins.



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a	1. 13,400	2. 1,280	3. 1 (Absent)	4. 1,840	5. 1,700
b	1. 11,670	2. 1,250	3. 9 (Absent)	4. 2,010	5. 1,450

FIG. 1A and B. Reproducibility is shown by microarray of 2 samples prepared independently from 1 individual. Similar intensities of the numbered probes are shown in the scanned images. **C.** The intensity of numbered spots.

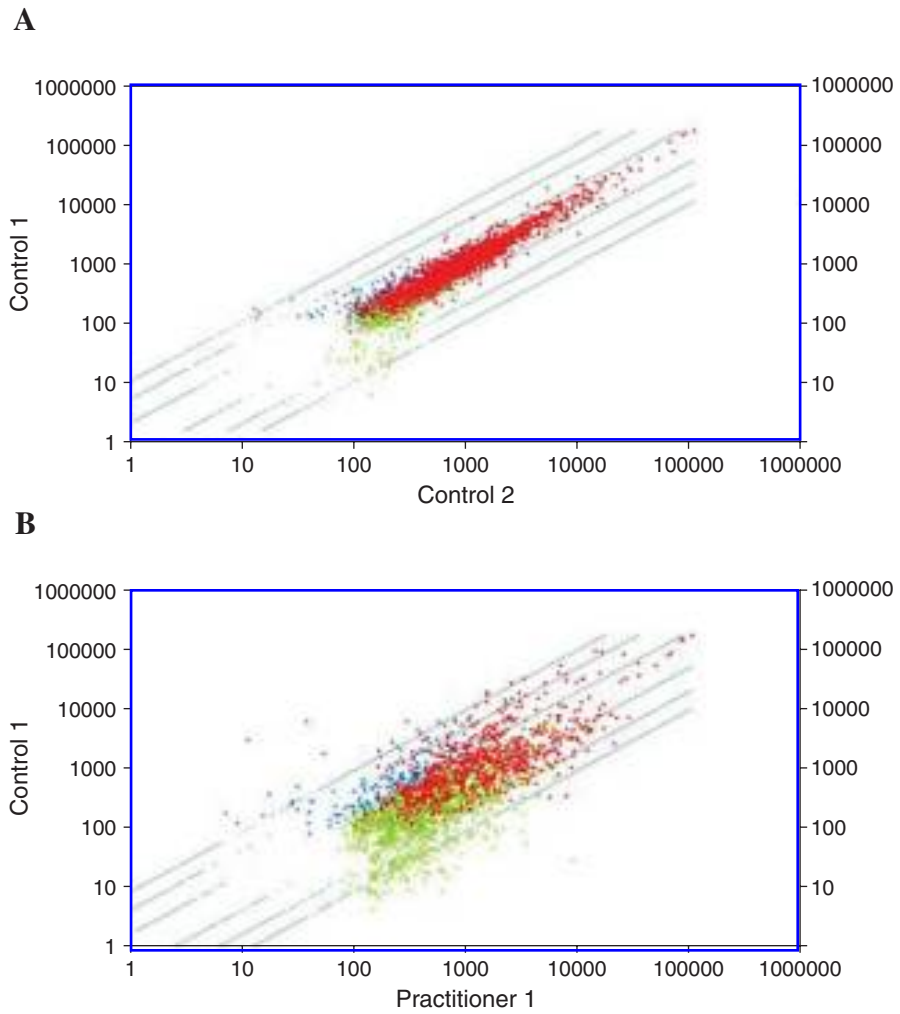


FIG. 2. Scatter-plot graph of microarray data from representative samples. All data points are on a 45° angle, confirming the accuracy of this technique. **A.** Scatter plot of control 1 (female, 35 years) vs. control 2 (female, 51 years). **B.** Scatter-plot of control 1 vs. Falun Gong practitioner (female, 41 years).

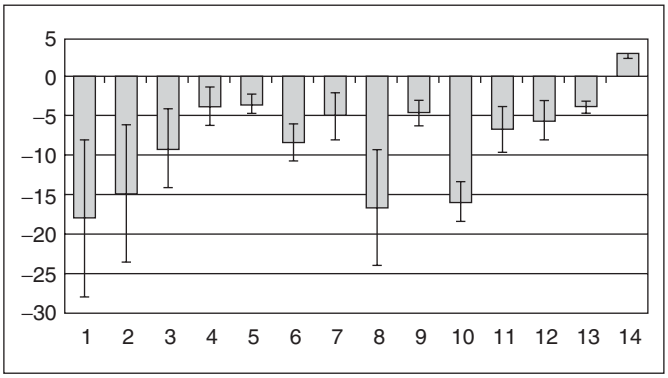
cRNA preparation

PMN cells were homogenized. Total RNA was extracted and purified with the Qiagen RNeasy kit (Qiagen, San Diego, CA). Five μg of total RNA were used in the first-strand cDNA synthesis with T7-d(T) 24 primer [GGCCAGTGAATTGTAATACGACTCACTATAGG GAGGCGG-(dT) 24] and Superscript II (GIBCO-BRL, Rockville, MD). The second-strand cDNA synthesis was carried out at 16°C by adding *Escherichia coli* DNA ligase, *E. coli* DNA polymerase I, and RNase H to the reaction, followed by T4 DNA polymerase to blunt the ends of newly synthesized cDNA. The cDNA was purified through phase lock gels (PLG)-phenol/chloroform extraction and ethanol precipitation. Using a BioArray High Yield RNA Transcript Labeling Kit (Ensue Diagnostics, NY), the purified cDNA was incubated at 37°C for 6 h in an *in vitro* transcription reaction to produce cRNA labeled with biotin.

Affymetrix chip hybridization

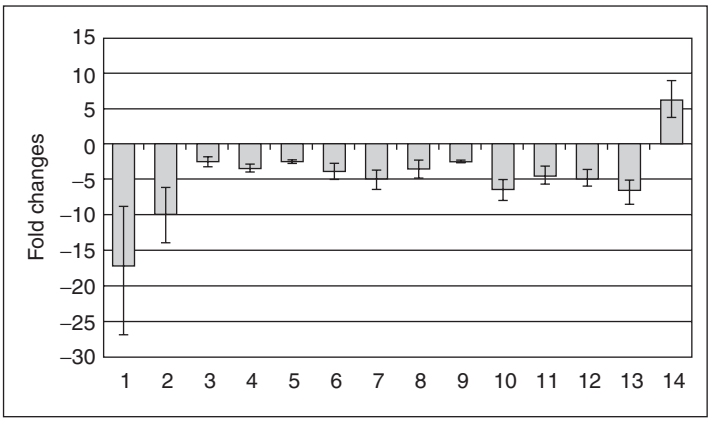
Hybridization was performed as described previously (Li et al., 2002). In brief, twenty μg of cRNA was fragmented by incubating in a buffer containing 200 mmol Tris-acetate (pH 8.1) at 95°C for 35 minutes. The fragmented cRNA was hybridized with a pre-equilibrated Affymetrix chip (Human Genome U95Av2) at 45°C for 14 h to 16 h. After the hybridization cocktails were removed, the chips were washed in a fluid station with low-stringency buffer (6× standard saline phosphate with EDTA, 0.01% Tween 20, and 0.005% antifoam) for 10 cycles (2 mixes per cycle) and a high stringency buffer (100 mg N-morpholino-ethanesulfonic acid, 0.1 mol/dm^3 NaCl, and 0.01% Tween 20) for 4 cycles (15 mixes per cycle) and stained with streptavidin phycoerythrin (SAPE). This process was followed by incubation with biotinylated mouse anti-avidin antibody and restaining with SAPE. The chips were scanned in an HP ChipScanner (Affymetrix, Santa Clara, CA) to detect hybridization signals.

A



1	ubiquitin gene	u49869
2	ubiquitin gene	X04803
3	ubiquitin conjugating enzyme	L22005
4	Parkin	AB009973
5	IAP homolog B	U37547
6	beta-spectrin	J05500
7	F-box, ubiquitin ligase	AF050254
8	hSIAH2, ubiquitin ligase	U76248
9	BRCA 1	L78833
10	RING protein	U69559
11	omithine decarboxylase antizyme	D78361
12	omithine decarboxylase antizyme	D78361
13	Catalase	AL035079
14	TRIM-containing RING protein	Y07828
15	proteasomal ATPases	AL050378

B



1	ribosomal protein S12	AA977163
2	ribosomal protein L30	L05095
3	ribosomal protein S1	u05589
4	ribosomal protein L18	L11566
5	ribosomal protein S4	m58458
6	ribosomal protein L21	u25789
7	ribosomal protein L28	u14969
8	ribosomal protein S20	L06498
9	ribosomal protein L7	x57958
10	ribosomal protein L31	X15940
11	ribosomal protein S24	m31520
12	ribosomal protein S11	X06617
13	ribosomal protein S17	M13932
14	ribosomal protein L35	AI541285

FIG. 3. The bar graph represents changes in gene expression > twofold in neutrophils from 6 Falun Gong (FLG) practitioners who have practiced FLG for at least 1 year (range, 1–5 years). Positive values indicate an increase in gene expression and negative values indicate a decrease. The solid bars represent the mean fold changes of the FLG practitioners in contrast to a pooled sample from 6 normal controls, and the error bars represent the variations per individual for each gene. **A.** Downregulation of UPP-related genes. **B.** Decrease of ribosomal genes. **C.** Alteration of stress-related and heat shock genes. **D.** Regulation of immunity-associated genes.

Data analysis

Hybridization data from text files were imported to a Microsoft Excel spreadsheet. Data analysis was performed to identify signals that were at least twofold different between FLG practitioners and normal control samples.

RNase protection assay

RNase protection assay (RPA) was used to confirm the alteration of some genes in the subjects, as previously described, using a manufacturer's protocol (Torrey Pines Biolabs, Houston, TX). RPA was performed as described previously (Feng et al., 1994; Garcia et al., 2003; Xia et al., 1999). Total RNA was extracted from neutrophils using a single-step method (Chomczynski and Sacchi, 1987). Two μg of total RNA from each sample was used for the RPA. The probes used were ubiquitin (BM172182, bp 146–456); I-309 (M57502, bp 1–250); defensin (AW468629, bp 1–300), and the H-APO-2c multi-probe set (BD Pharmingen, San Diego, CA). The density of protected bands was determined by densitometry using the public domain NIH Image program (developed at the National Institutes of Health and available on the Internet at rsb.info.nih.gov/nih-image/).

ELISA for measurement of DNA fragmentation

Cell death of neutrophils was assayed with a kit from Roche (Indianapolis, IN). In brief, 10^4 neutrophils in 200 mL of RPMI 1640 with 3% FBS were cultured in 96-well plates at 37°C and harvested at different time points. After incubation, the cells were pelleted by centrifugation and resuspended in lysis buffer. After lysis, intact nuclei were pelleted by centrifugation. Nucleosome-containing supernatant was transferred to the streptavidin-coated plate and incubated with anti-histone (biotin-labeled) and anti-DNA (peroxidase-conjugated) antibodies to form an antibody-nucleosome complex. After washing the plate, samples were incubated with peroxidase substrate (ABST) and the absorbance was measured at 405 nm.

RESULTS AND DISCUSSION

The Affymetrix Human Genome U95 (HG-U95) set, consisting of 5 GeneChip® arrays, contains almost 63,000 probe sets interrogating approximately 54,000 clusters derived from the UniGene database. The HG-U95Av2 array repre-

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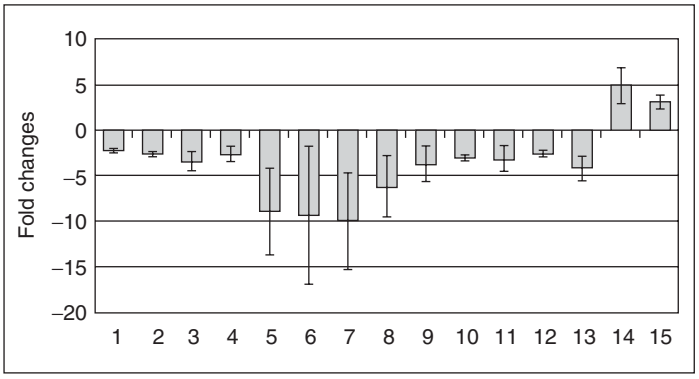
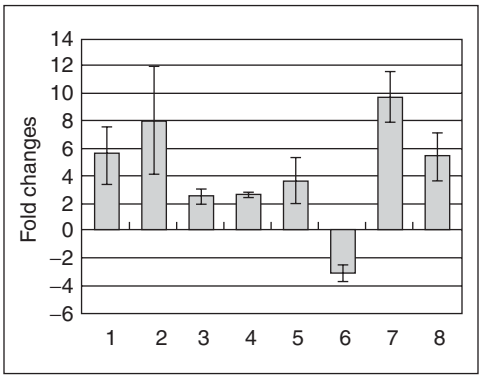


1	LIM protein (IPP)	U49957
2	RAD52 (Radiation resistance)	L33262
3	ERCC2	X52221
4	excision repair protein (ERCC1)	M13194
5	selenium-binding protein (hSBP)	U29091
6	transformation-related protein	L24521
7	biliverdin-IXbeta reductase I	D32143
8	nucleotide-disulphide oxidoreductase	AB018293
9	amine oxidase	AF047485
10	glutathione transferase	J05459
11	glutathione peroxidase	X71973
12	multidrug resistance-associated protein	U83660
13	E16 mRNA, amino acid transporter	M80244
14	heat-shock protein HSP70B	X51757
15	heat-shock protein hsp40-3	AF088982

D



1	tyk2 tyrosine kinase	x54637
2	pSK1 IGRAF-1	u05875
3	oligoadenylate synthetase	m87284
4	IFN-gamma	x13274
5	interferon regulatory factor 7B	u53831
6	sarcolectin	aj238246
7	neutrophil defensin-3	L12691
8	I-309	M57506

FIG. 3. *Continued.*

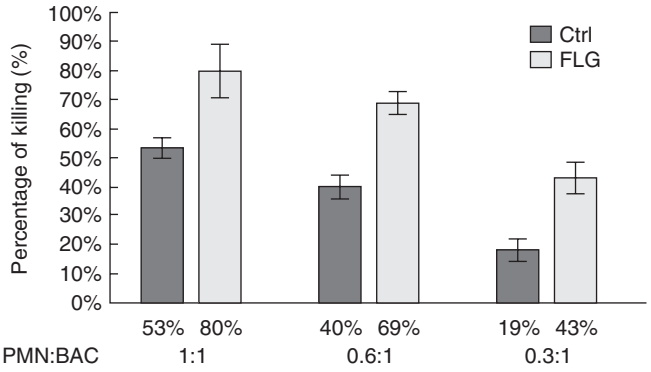
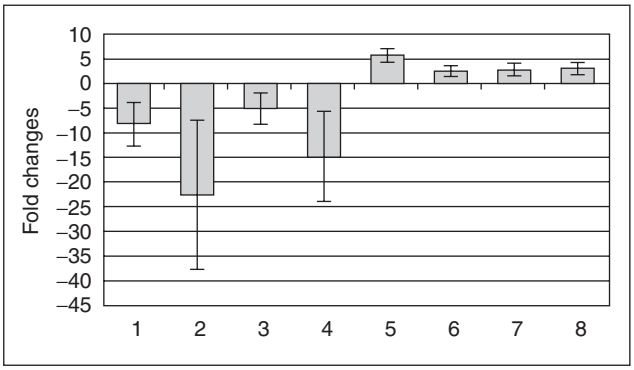


FIG. 4. *Staphylococcus aureus* was used as the target organism in the bactericidal assay. Maximal neutrophil bactericidal activity was measured by the decrease in the number of viable bacteria after incubation of bacteria with neutrophils. The bactericidal activity was expressed as the percentage of killed bacteria. The solid bars represent the mean of 6 Falun Gong (FLG) practitioners or 6 normal controls and the values from each subject are generated based upon 3 independent experiments. Error bars represent the individual variations in each experimental condition. Bactericidal activity exerted by polymorphonuclear leukocytes (PMN) was significantly increased in FLG groups in comparison with normal controls and statistical significance was found in all effectors/target ratios ($p < 0.0001$).

sents approximately 12000 full-length genes. Among 12000 genes examined, 250 genes were consistently changed, with 132 genes downregulated and 118 genes upregulated in 6 FLG practitioners in contrast to 6 normal healthy controls. In order to measure the variation in gene expression between different preparations, separately prepared samples from one subject were used to hybridize the slides, and signals were scanned and recorded as described in the Materials and Methods section. As shown in Figure 1, every spot is rectangular and has virtually the same size and similar signal intensities in separate experiments, indicating reproducibility. A scatter-plot of 2 different controls (35-year-old female and 51-year-old male) demonstrates a line of identity with very few genes showing different levels of expression (Fig. 2A). Fewer than 50 genes showed a twofold or great difference in expression between 2 normal controls. When a control (35-year-old female) was compared with a practitioner (39-year-old female), significant numbers of altered genes were seen (Fig. 2B). Since the difference in controls was not significant, the pooled RNA sample from 6 normal controls was used as a reference for individual samples from practitioners. The fold changes between RNA of each practitioner and the pooled RNA sample (reference) of the 6 controls is shown as mean ± standard deviation (SD) in all figures.

Among the changed genes, the most interesting groups of genes are functionally associated with protein degradation and synthesis (Figs. 3A,B). The major mechanism used by eukaryotic cells for disposing of misfolded or damaged proteins present in the cell is an ubiquitin-dependent protein degradation pathway (UPP) (Glickman and Ciechanover, 2002). The UPP modification of many cellular proteins plays an important role in a variety of basic cellular processes. This system has been recently implicated in the pathogenesis of many disorders, including genetic diseases, neuronal degenerative diseases, cancers, muscle wasting, diabetes, hypertension, sepsis, autoimmune diseases, inflammation, and aging-related disorders.

The main components of the UPP are ubiquitin protein, ubiquitin-like proteins, and 3 major enzymes: E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme), and E3 (ubiquitin ligase) (Glickman and Ciechanover, 2002). RING (for *really interesting new gene*) finger proteins are a group of proteins that have recently been linked to a specific function (Joazeiro and Weissman, 2000). We found that 3 classes of genes including ubiquitin, E2, and E3 are significantly downregulated. Tripartite motif (TRIM)-containing RING protein (Reymond et al., 2001), which belongs to the RING-B-box-coiled-coil (RBCC) family, is the only upregulated RING-finger-containing protein among all RING finger proteins we identified, and the biologic meaning remains to be further determined. In addition, an ubiquitin-independent protein degradation enzyme, ornithine decarboxylase antizyme (Coffino, 2001), was significantly decreased as well. Downregulation of the UPP system may result from lowering of the metabolic rate, rather than being the direct consequence of meditation.



1	Bcl-xL	Z23115
2	BCL2	AB079221
3	pim-1 oncogene	M16750
4	FKBP38	L37033
5	TRADD	L41690
6	BID	AF042083
7	interleukin-1 beta convertase	M87507
8	interleukin-1b-converting enzyme 2	U86214

FIG. 5. Alteration of apoptosis-related genes. The bar graph represents changes in gene regulation > twofold in neutrophils from 6 Falun Gong (FLG) practitioners who have practiced FLG for at least 1 year (range, 1–5 years). The solid bars represent the mean fold changes in contrast to a polled sample from 6 normal controls and the error bars represent individual variations for each gene.

Ribosomal proteins are very important components of protein synthesis. Downregulation of 10 out of 11 genes for ribosomal proteins suggests that protein synthesis might also be lowered. Ribosomes are the molecular machines that manufacture proteins (Maguire et al., 2001). Downregulation of both genes for ribosomal proteins and genes for protein degradation may lead to reduced protein turnover. In correlation with downregulation of protein degradation and synthesis, the genes coding for proteins involved in DNA repair, cellular stress, and antioxidant enzymes are also lowered (Fig. 3C). Decreases of those stress-associated key enzymes, along with other stress-responsive genes, may implicate limited oxidative production and macromolecular damage. These genes, which include RAD52 (for radiation resistance) and ERCC (for excision repair), mammalian homologues of yeast genes, are involved in DNA repair and recombination. RAD52 and ERCC group genes are required for the repair of DNA damage induced by ionizing radiation and a variety of stressors (Symington, 2002; Tuteja and Tuteja, 2001). Unexpectedly, two heat shock proteins, HSP70 and HSP40-3, are significantly increased, in contrast to other downregulated stress-responsive genes. It would be interesting to investigate whether some of the stress-responsive genes may be beneficially upregulated for protection (Latchman, 2001).

It has been documented that spiritual practice and mind–body approaches may enhance the effectiveness of the immune system and, furthermore, are clinically effective in treating a variety of diseases (Coker, 1999; Jones, 2001; Shang, 2001). Psychologic stress and negative emotions can drastically intensify infection through immune dysregulation (Kiecolt-Glaser et al., 2002). It could be speculated that immunity-associated genes may also be altered in FLG practitioners. As shown in Figure 3D, interferon (IFN)-γ and IFN-related and IFN-regulated genes [e.g., pSK1 IFN-γ factor 7B (8.3 ± 4.9 folds)], were increased, excepting the IFN inhibitor sarcolectin (Kaba et al., 1999). Interestingly, an IFN-inducible gene, oligoadenylate synthetase, was upregulated $\sim \times 3$ (Samuel, 2001). Antimicrobial peptides are a prevalent group of molecules in the host defense system. Defensin 3, a cationic peptide involved in nonoxidative antimicrobial mechanisms (Horuk et al., 1998; Lee et al., 2000), was increased in all of the FLG practitioners examined. I-309, a chemotactic cytokine that also functions as an HIV coreceptor (Horuk et al., 1998; Lee et al., 2000), was significantly increased in the FLG practitioners. The significant upregulation of I-309 may suggest alteration of chemokine-mediated cellular migration and immunity. Induction of IFN-γ transcripts may represent another beneficial change in immunity, since IFN-γ is an important cy-

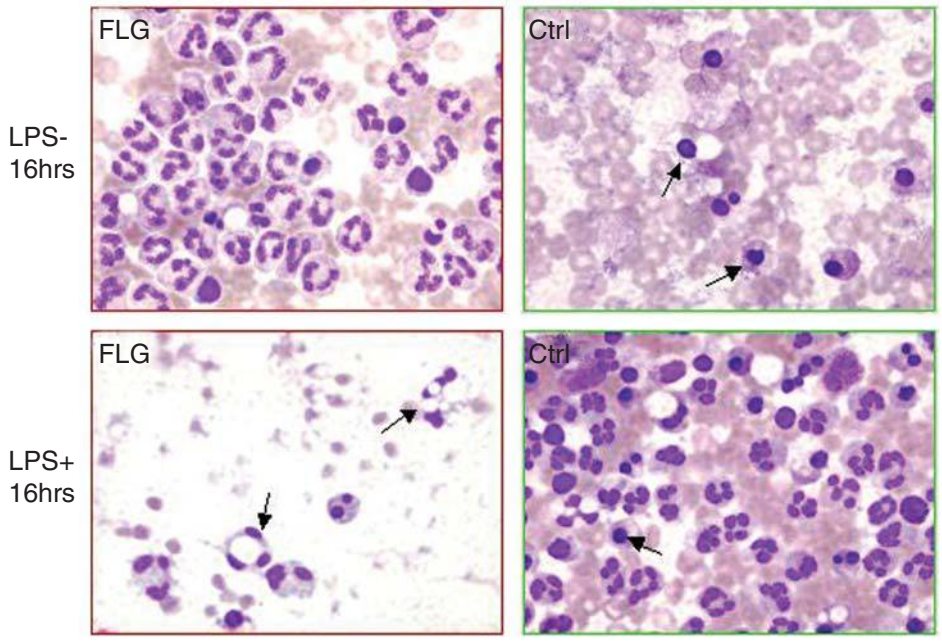


FIG. 6. Micrographic photo of altered neutrophil apoptosis in the presence (LPS plus, LPS+) and absence of lipopolysaccharide (LPS) (LPS minus, LPS−) after 16-hour culture. Neutrophils were cultured for 16 hours in the absence (upper panel) and presence (lower panel) of LPS (25 ng/mL). Wright-stained at different timepoints and examined by light microscopy. Arrows indicate shrunken neutrophils with chromatin condensation, rounded nuclear profiles, and presence of cytoplasmic vacuolization. Most of the neutrophils from a Falun Gong practitioner were alive without apoptosis in the absence of LPS (upper and left panels); however, most of the neutrophils, when stimulated with LPS were apoptotic (lower and left). In contrast, neutrophils from a normal control without LPS stimulation were apoptotic (upper and right); however, neutrophils, when stimulated with LPS, were alive (lower and right). Wright-Giemsa stain. Original magnification: ×400.

tokine in the host defense against infection by viral and microbial pathogens (Samuel, 2001). Functional evidence of gained innate immunity was further supported by increased neutrophil phagocytosis, indicating a functional correlation between gene regulation and cellular activity (Fig. 4).

Immunity enhancement by mind–body practice may be beneficial, but the immune response is a double-edged sword, and activation of the immune system could be detrimental to the host when the immune response is sustained. Delayed cell death may suggest a prolonged inflammatory process, while enhanced immunity may be harmful to the host. The following data may indicate that mind–body-mediated regulation may represent a balance within. We identified that the apoptosis-related-genes *Bcl-2*, *Bcl-xL*, and *FKBP* 38 were significantly downregulated (Fig. 5). *Bcl-2* and *Bcl-xL* are the antiapoptotic members of the Bcl family (Adams and Cory, 2001; Zimmermann and Green, 2001), and a decrease of those antiapoptotic genes may be responsible for the acceleration of neutrophil apoptosis when stimulated with LPS as shown in Figures 6 and 7. The accelerated neutrophil apoptosis might lead to a rapid resolution of inflammation, resulting in limitation of self-injuries mediated by enhanced immunity, since delayed neutrophil apoptosis has been linked to a variety of chronic inflammatory injuries (Smith, 1994; Webb et al., 2000). However, prolonged survival of neutrophils from practitioners in the absence of LPS may represent a homeostatic modulation in a normal condition. We hypothesized that the prolonged neutrophil lifespan may result from lowering of meditation-mediated metabolic rate since oxidative stress, instead of apoptotic genes, is the main factor responsible for spontaneous cell death of neutrophils in biologic conditions (Akgul et al., 2001; Kasahara et al., 1997). It was reported that lower serum lipid peroxide levels might be associated with other meditation-mediated stress reduction (Schneider et al., 1998). In addition, a decrease of *FKBP* 38 may favor neutrophil apoptosis, since FKBP 38 is a mitochondrial FK506-binding protein which inhibits apoptosis by anchoring Bcl-2 and Bcl-xL to mitochondria (Shirane and Nakayama, 2003) stimulated by LPS. In contrast to antiapoptotic genes, proapoptotic genes were upregulated, including TNFR-1 associated protein, BH3 interacting domain death agonist, interleukin-1β-convertase, and interleukin-1β-converting enzyme 2.

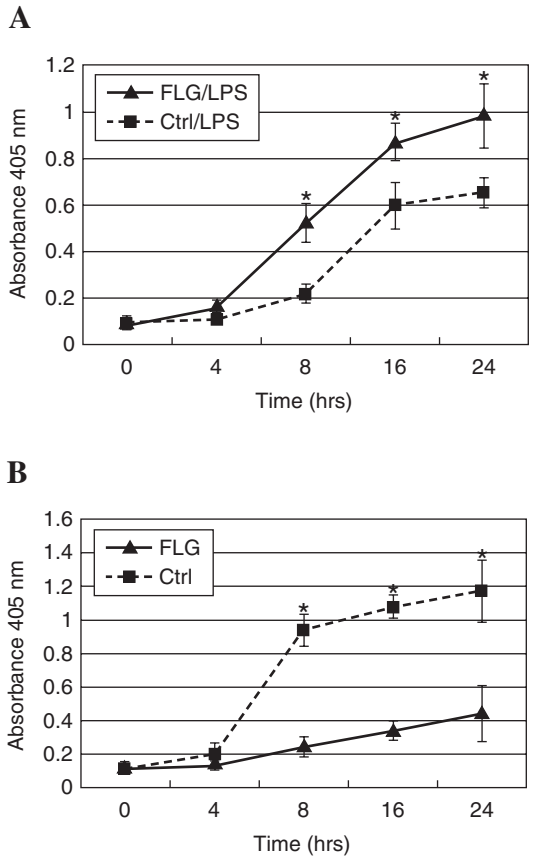


FIG. 7. Time-dependent neutrophil apoptosis. In the presence of LPS **(A)**, the neutrophils from Falun Gong (FLG) practitioners were apoptotic significantly faster than the neutrophils from normal controls at 8, 16, and 24 hours (Ctrl). However, in the absence of lipopolysaccharide (LPS) **(B)**, the neutrophils from FLG practitioners survived much longer than the ones of normal controls at the matched timepoints. * Indicates a significant difference between the means of groups, $P < 0.01$, analyzed by Student's t test. The data were generated from FLG practitioners ($n = 6$) relative to control PMNs ($n = 6$) with timepoints at 0, 2, 4, 8, and 16 hours.

LPS is a proinflammatory agent and can stimulate cells to express multiple cytokines and growth factors which may be involved in the delay of apoptosis of proinflammatory cells; this is well documented in the literature (Smith, 1994). Delayed neutrophil apoptosis has been linked to a variety of chronic inflammatory injuries (Webb et al., 2000). The alteration of the apoptosis-related genes may be responsible for the accelerated LPS-stimulated apoptosis seen in neutrophils from FLG practitioners. Conversely, the accelerated neutrophil apoptosis may lead to a rapid resolution of inflammation, resulting in limitation of self-injury mediated by enhanced immunity (Matzinger, 2002; Medzhitov and Janeway, 2002; Simpson and Hines, 2002). The survival of neutrophils from FLG practitioners in the absence of LPS was significantly prolonged and may also result from lowering of the meditation-mediated metabolic rate, since oxidative stress, not apoptotic genes, is the main factor responsible for spontaneous cell death in biological conditions (Akgul et al., 2001; Kasahara et al., 1997). It has been demonstrated that lower serum lipid peroxide levels might be associated with stress reduction mediated by meditation (Schneider et al., 1998).

In order to confirm microarray data, RPA was performed. Most of the changed genes could not be tested by RPA because of the limited amount of RNA. The ubiquitin gene was selected for confirmation by RPA due to its unique role in protein metabolism. Both I-309 and defensin were chosen because they play an important role in both innate and adaptive immunity. A group of antiapoptotic genes was selected because altered apoptosis was observed in the practitioners. As shown in Figure 8A, ubiquitin was barely de-

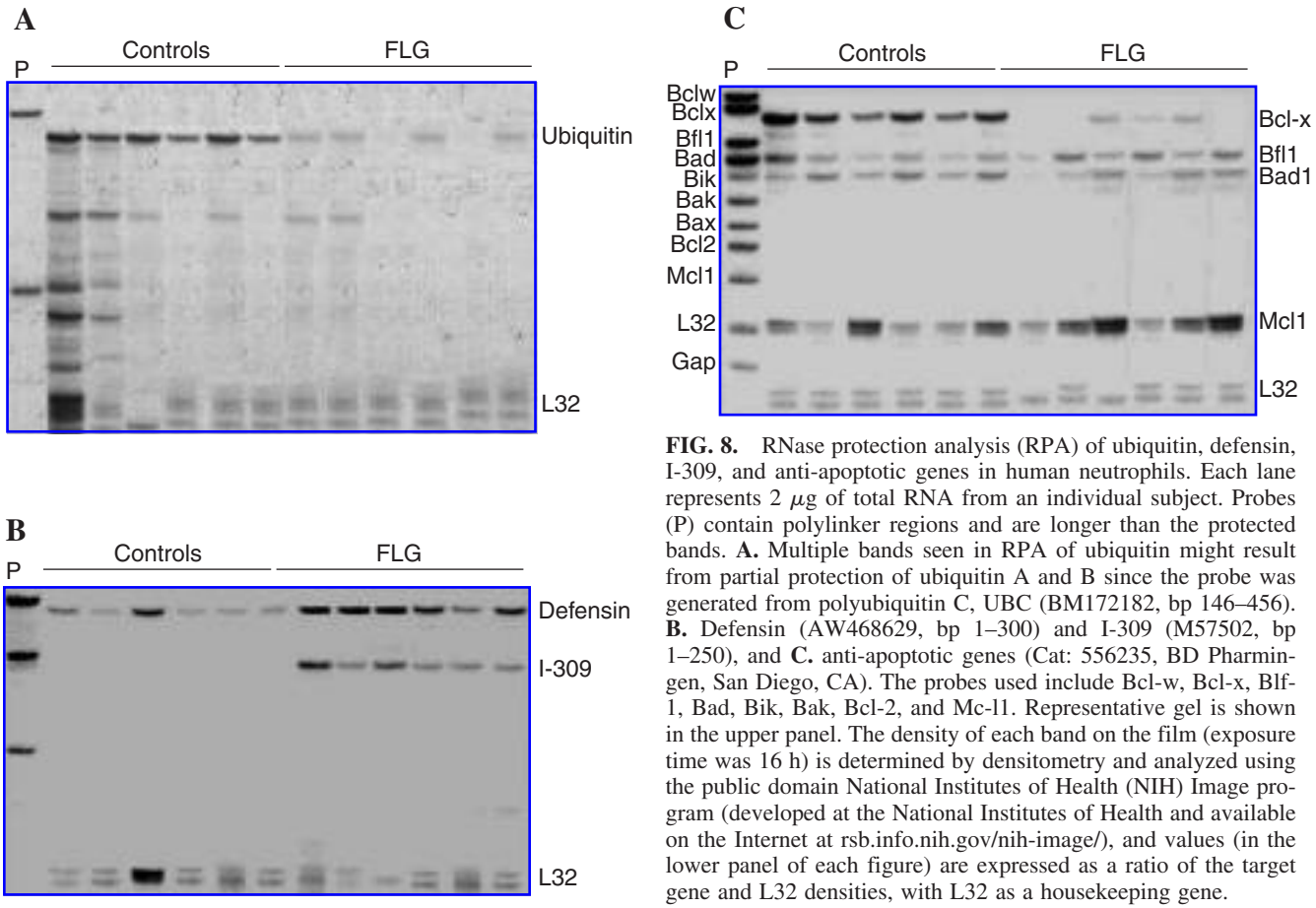


FIG. 8. RNase protection analysis (RPA) of ubiquitin, defensin, I-309, and anti-apoptotic genes in human neutrophils. Each lane represents 2 μg of total RNA from an individual subject. Probes (P) contain polylinker regions and are longer than the protected bands. **A.** Multiple bands seen in RPA of ubiquitin might result from partial protection of ubiquitin A and B since the probe was generated from polyubiquitin C, UBC (BM172182, bp 146–456). **B.** Defensin (AW468629, bp 1–300) and I-309 (M57502, bp 1–250), and **C.** anti-apoptotic genes (Cat: 556235, BD Pharmingen, San Diego, CA). The probes used include Bcl-w, Bcl-x, Blf-1, Bad, Bik, Bak, Bcl-2, and Mc-l1. Representative gel is shown in the upper panel. The density of each band on the film (exposure time was 16 h) is determined by densitometry and analyzed using the public domain National Institutes of Health (NIH) Image program (developed at the National Institutes of Health and available on the Internet at rsb.info.nih.gov/nih-image/), and values (in the lower panel of each figure) are expressed as a ratio of the target gene and L32 densities, with L32 as a housekeeping gene.

tected in all 6 FLG practitioners in contrast to the controls ($p < 0.01$) (Table 1). The multiple bands seen below the main signal may result from partial protection of the ubiquitin probe by 2 other types of ubiquitin transcripts (B and C) with high homology. Defensin was detected in the controls, which is consistent with the findings by others, and both defensin and I-309 were significantly elevated in FLG practitioners, as observed in the microarray assay as shown in Figure 8B. The probe set containing multiple antiapoptotic genes was used to determine the expression level of *Bcl-x*, which was identified as a changed gene by array. *Bcl-x* expression is drastically reduced in FLG practitioners, while the other antiapoptotic genes, such as *Bfl1*, *Bad1*, and *Mcl1*, were relatively unchanged (Fig. 8C). Significant downregulation of *Bcl-x* in FLG practitioners ($p < 0.02$) validated the microarray data. Although microarray data showed that *Bcl-2* was markedly reduced in FLG practitioners in contrast to controls, *Bcl-2* was not detectable in either controls or FLG practitioners by RPA. This inconsistency may be due to the difference in sensitivity of microarray versus the RPA.

Among 12000 genes tested in the Affymetrix chip, about 200 genes were consistently altered in the FLG practitioners, and we have discussed some of the changed genes. However, most of the altered genes cannot be discussed due to limited space, and the data are available upon request.

Downregulation of the ubiquitin system in the meditation practitioners may be explained by two possible mechanisms: (1) substrate-dependent downregulation, since "garbage disposal" UPP was downregulated, with decreased metabolic wastes resulted from metabolic reduction; or (2) meditation-mediated cellular activities directly leading to transcriptional arrest of UPP gene expression and downregulation of UPP. Although there are no studies to support that downregulation of the UPP system may be associated with a healthy phenotype in *Qigong* practitioners, some research with caloric restriction (CR) in rodents as well as primates may

TABLE 1. COMPARISON OF GENES SIGNIFICANTLY ALTERED IN FALUN GONG (FLG) PRACTITIONERS AS ASSAYED BY RNASE PROTECTION ASSAY (TARGET GENE, L32)

Gene	*Control* (n = 6)	*FLG* (n = 6)	p
Ubiquitin	1.11 ± 0.15	0.42 ± 0.08	0.01
Defensin	0.35 ± 0.06	1.47 ± 0.58	0.03
I-309	0.10 ± 0.02	0.39 ± 0.20	0.03
Bcl-x	5.54 ± 1.81	5.59 ± 2.17	0.02

shed some light on such a possibility, as CR is the only known method to delay the aging process and extend maximal lifespan in rodents as well as in primates (Lane et al., 2001; Roth et al., 2001). Such extension of lifespan is thought to involve metabolic reduction and downregulation of UPP activity (Lee et al., 1999), supporting the notion that metabolic reduction might lead to shrinkage of this "garbage disposal." Moreover, it was also found that CR animals develop significant resistance to stress and disease (Raffoul et al., 1999; Yu and Chung, 2001). A dramatic decrease of the ubiquitin protein degradation system in meditation practitioners suggests that this system may function as a molecular sensor of metabolic conditions. Interestingly, ubiquitin was characterized as a stress-inducible protein (Bond and Schlesinger, 1985; 1986).

CONCLUSIONS

Although our findings may establish some molecular cellular links to healthy phenotypes in *Qigong* practitioners, many more questions remain to be addressed. Do all FLG practitioners display the same genomic profile? What is the precise biologic meaning of gene regulation in neutrophils? Does the length of time of FLG practice correlate with changes in gene expression? Do other mind–body practitioners have similar changes in gene expression? Do mononuclear cells show similar changes? Could our findings be clinically useful? Although the mechanisms and generalizability remain unclear, our pilot study provides the first evidence suggesting that *Qigong* practice may exert effects on immunity, metabolic rate, and cell death, possibly through transcriptional regulation.

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