

RAPID COMMUNICATION

Identification of Potential mRNA Biomarkers in Peripheral Blood Lymphocytes for Human Exposure to Ionizing Radiation

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Since early in the Atomic Age, biological indicators of radiation exposure have been sought, but currently available methods are not entirely satisfactory. Using cDNA microarray hybridization to discover new potential biomarkers, we have identified genes expressed at increased levels in human peripheral blood lymphocytes after *ex vivo* irradiation. We recently used this technique to identify a large set of ionizing radiation-responsive genes in a human cell line (*Oncogene* 18, 3666–3672, 1999). The present set of radiation markers in peripheral blood lymphocytes was identified 24 h after treatment, and while the magnitude of mRNA induction generally decreased over time, many markers were still significantly elevated up to 72 h after irradiation. In all donors, the most highly responsive gene identified was *DDB2*, which codes for the p48 subunit of XPE, a protein known to play a crucial role in repair of ultraviolet (UV) radiation damage in DNA. Induction of *DDB2*, *CDKN1A* (also known as *CIP1/WAF1*) and *XPC* showed a linear dose–response relationship between 0.2 and 2 Gy at 24 and 48 h after irradiation, with less linearity at earlier or later times. These results suggest that relative levels of gene expression in peripheral blood cells may provide estimates of environmental radiation exposures.

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INTRODUCTION

As concerns about nuclear waste and other sources of manmade radiation rise, it is of increasing interest to develop biological markers that can identify exposed individuals in human populations. The methods used previously to estimate exposures after nuclear accidents include scoring chromosome aberrations (1) in peripheral blood lymphocytes (PBL), glycophorin A-based somatic mutation assays in erythrocytes (2), and electron spin resonance of tooth enamel (3). With recent developments in high-throughput gene expression screening, it may be possible

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to develop gene expression profiles in human PBL that correlate with the timing and dose of radiation exposures. The identification of such a gene set would enable more rapid and noninvasive testing of potentially exposed populations. As a first step toward defining such a gene set, we describe a set of genes induced in human PBL up to 3 days after *ex vivo* irradiation with doses from 0.2 to 2 Gy.

MATERIALS AND METHODS

Separation and Irradiation of Human Peripheral Blood Lymphocytes

Human blood from normal healthy donors was obtained from the NIH blood bank (Department of Transfusion Medicine), and within 30–60 min of drawing, the components were separated by centrifugation on a Lymphoprep (Nycomed) density gradient. The buffy coat layers were washed in phosphate-buffered saline and resuspended at a density of 0.5 to 1 × 10⁶ cells per milliliter in RPMI 1640 medium supplemented with 10% heat-inactivated (56°C for 45 min) fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified, 5% CO₂ atmosphere. PBL were allowed to equilibrate to culture conditions for 45–60 min, then irradiated at approximately 60 cGy/min with total doses of 20 cGy to 2 Gy using a Mark I-68 ¹³⁷Cs γ-ray source (J. L. Shepherd and Associates, Inc.) with lead attenuators in place.

Microarray Hybridization and Analysis

A total of 100–200 µg of whole-cell PBL RNA was labeled and hybridized to 6728 element microarrays as described previously (4). In brief, probes were prepared by PCR amplification of IMAGE consortium clones and arrayed on poly-L-lysine-coated glass slides. Fluorescently labeled cDNA was prepared from control and γ-irradiated PBL whole-cell RNA by a single round of reverse transcription (BRL Superscript II) in the presence of fluorescent dNTP (Cy3 dUTP or Cy5 dUTP, Amersham). Probes and targets were hybridized together for 16 h in 3× SSC at 65°C in the presence of the blockers human *COT1* DNA, yeast tRNA and polydeoxyadenylic acid. Hybridized slides were washed at room temperature once in 0.5× SSC, 0.01% SDS for 5 min, and again in 0.06× SSC for 5 min. Cy3 and Cy5 fluorescences were scanned using a laser confocal microscope, and images were analyzed using the DeArray program to calibrate relative ratios and develop confidence intervals for their significance (5). The ratios were normalized to those of a set of 88 internal controls (6) with a theoretical ratio of 1.0. The variance in the house-keeping set was used to determine the significance of expression changes after irradiation.

Measurement of Gene Induction

Probes for single-sequence hybridization were PCR-amplified from the plasmids used to print the microarrays with universal primers AEK

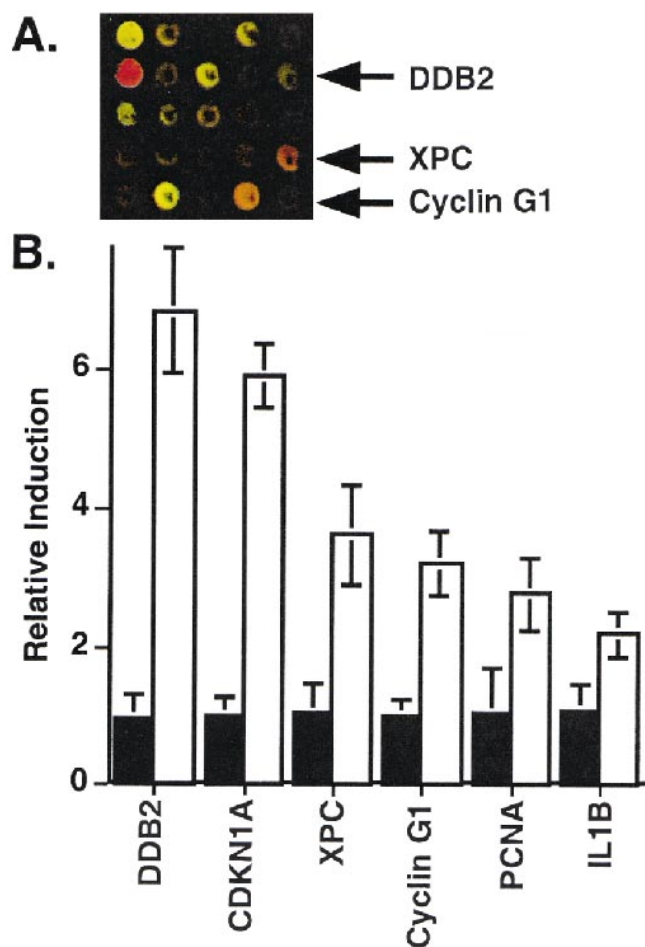


FIG. 1. Panel A: Section of microarray hybridized to RNA from unirradiated (shown in green) and 2-Gy γ -irradiated (red) human peripheral blood lymphocytes harvested 24 h after irradiation. The arrows indicate several of the up-regulated markers (appearing as reddish spots) characterized here. Panel B: Induction of markers in PBL of eight independent donors. The black bars represent the mean levels in untreated PBL after normalization to the levels in the first donor. The white bars show the mean fold induction above untreated levels 24 h after 2 Gy γ rays in these donors. Error bars are standard deviations.

M13F: GTTGTAACACGACGGCCAGTG and AEK M13R: CACA-CAGGAAACAGCTATG. RNA was extracted from PBL cultures using a modified guanidine thiocyanate technique (7). Serial dilutions of RNA were immobilized on nylon membranes, hybridized to probes at 55°C in Hybrisol I (Oncor), and washed under standard conditions as described previously (4). Hybridization was quantified on a PhosphorImager (Molecular Dynamics), and relative signal levels, normalized to the polyA content of each sample, were determined using the RNA-Think program (8). With this approach, differences of 1.5-fold or more can be measured reliably (8, 9) and agree well with results of RNase protection assays (10).

RESULTS AND DISCUSSION

RNA harvested from exposed and control quiescent PBL 24 h after exposure to 2 Gy *ex vivo* γ rays was hybridized to a cDNA microarray containing 6728 cDNA sequences (Fig. 1A). Analysis of this microarray revealed 48 genes that were significantly up-regulated compared to their levels

in the untreated control. In the same sample, only 7 genes were significantly down-regulated by the γ -ray treatment. The complete results of this experiment can be accessed on our web site at <http://rex.nci.nih.gov/RESEARCH/basic/lbc/fornace.htm>. Among the induced genes were several cytokines and growth factors, as might have been expected. These included *IL1A*, *IL1B*, *IL6* and small induced cytokine A4 (*SCYA4*). Genes previously associated with stress response signaling or DNA repair, such as *CDKN1A* (also known as *CIP1/WAF1*), *XPC*, *DDB2* and *PCNA*, were also found to be induced.

A number of the genes detected by the microarray as significantly induced in PBL after irradiation were selected for further study (Fig. 1A). In a previous study using microarray analysis of irradiated cell lines, we found greater than 95% agreement between gene induction as measured by microarray and by single-probe hybridization (4). In the current study, RNA from PBL of an individual donor was hybridized to the microarray; then the 2-Gy irradiation with harvest of RNA after 24 h was repeated with PBL populations isolated from a number of independent donors to gauge the extent and uniformity of responses among different individuals. Eight of 10 genes tested showed responses in independent samples from different donors similar to those seen with the microarray in the first individual. Quantitative single-probe hybridizations with *DDB2*, *CDKN1A*, *XPC*, cyclin G1 (*CCNG1*), *PCNA* and *IL1B* confirmed robust induction of these transcripts 24 h after irradiation in all donors tested (Fig. 1B). Furthermore, there was less than twofold interindividual variation in the levels of these transcripts in unirradiated PBL, indicating that distinct ranges of uninduced transcript levels might be defined for these markers. This would be very important for the use of these genes as markers of exposure, as unirradiated control samples would likely not be available for each potentially exposed individual.

These initial experiments were all carried out using unstimulated, quiescent PBL populations, the better to mimic the conditions of peripheral blood cells as they might be irradiated during an *in vivo* exposure. As many of the genes, such as *CDKN1A* or *DDB2*, found to respond to *ex vivo* irradiation have previously been associated with responses to DNA damage, it was possible that the quiescent nature of the irradiated cell population could be masking a potentially larger response. We hypothesized that if the cells were stimulated to divide, latent DNA damage that was tolerated in the nondividing cells might trigger a larger signal, resulting in amplification of the stress gene response. In an attempt to amplify such a DNA damage response, phytohemagglutinin and conditioned growth medium were used to stimulate the PBL 24 h after irradiation. While the stimulation of the PBL was successful, as indicated by the large induction of the transcript for interferon γ -induced protein 10 (Fig. 2A), the lymphocyte stimulation alone had no effect on the level of *DDB2* mRNA in unirradiated cells. This marker of lymphocyte stimulation was

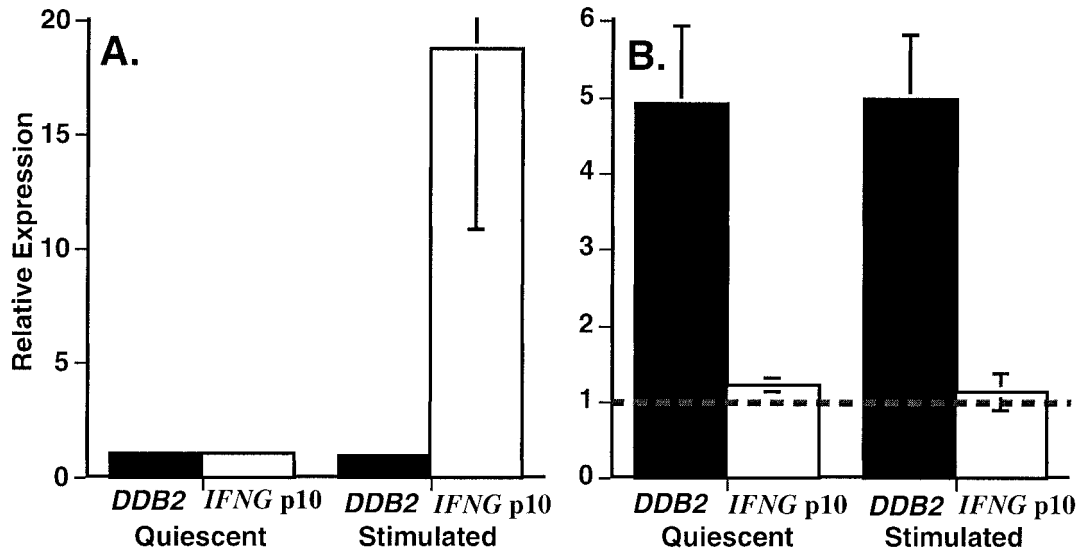


FIG. 2. Panel A: PBL stimulated to divide and harvested 24 h later showed a large increase in mRNA levels of *IFNG* p10, a cytokine-induced transcript, indicating the cells' response to the mitogenic stimulation (white bars). Levels of *DDB2* (black bars), however, remained unaltered from those in quiescent cells. The bars are the means of measurements in three independent donors, and error bars are standard deviations. (Variations in the expression levels of both genes in the quiescent state and of *DDB2* in the stimulated state were so small as not to be visible on the graph.) Panel B: Relative γ -ray induction above the background level of *DDB2* (black bars) and *IFNG* p10 (white bars) in quiescent or stimulated PBL. The dashed line indicates the level of gene expression in unirradiated cells. The bars are the mean of measurements in three independent donors, and error bars are standard deviations.

not further induced by 2 Gy γ rays, nor was the radiation induction of *DDB2* above baseline levels altered in the stimulated lymphocytes compared to those in the quiescent state (Fig. 2B). While it is still possible that stimulation of lymphocytes from irradiated blood samples will reveal ad-

ditional markers not indicated in our study of quiescent PBL, this technique does not appear to enhance the expression of the markers in this study. This finding further implies that although the PBL are not progressing through the cell cycle, their systems for sensing and responding to DNA damage are nonetheless active.

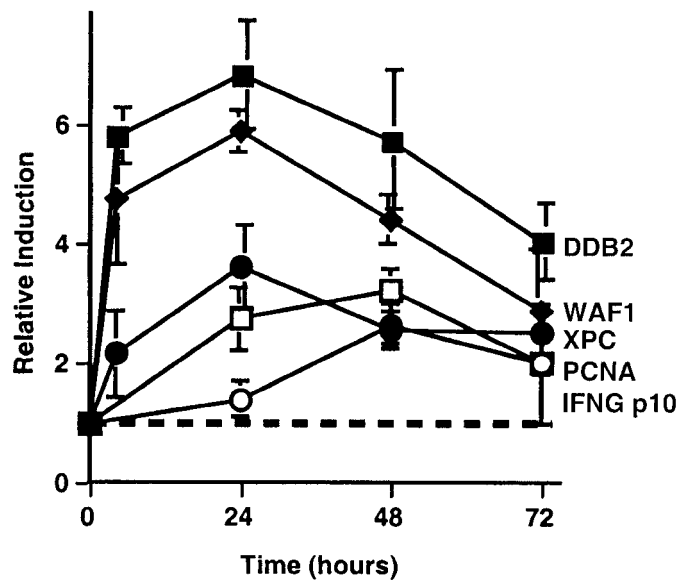


FIG. 3. Time course of marker levels after 2 Gy γ radiation delivered *ex vivo*. Each point is the mean of relative induction above background in three or four independent donors for *DDB2* (■), *CDKN1A* (WAF1) (◆), *XPC* (●), *PCNA* (□) and *IFNG* p10 (○). RNA was harvested from lymphocytes after 1, 2 or 3 days of incubation. Error bars are standard errors of the mean. The dashed line shows the basal level of expression in untreated PBL.

In previous studies using cultured cell lines, peak induction of genes such as *CDKN1A* (4) occurred at 4 h after irradiation with high doses of ionizing radiation, and at even earlier times with doses under 50 cGy (11). Although this time may be too short to be useful in potential exposure situations, we measured induction of *CDKN1A*, *DDB2* and *XPC* at 4 h after irradiation. Contrary to what might have been expected from previous results with cultured cell lines, the magnitude of induction of all three of these genes by 2 Gy was less after 4 h than that after 24 h (Fig. 3). It may be that the maximal stress response can be mounted more swiftly in the rapidly proliferating cell lines, while induction in the quiescent cell populations requires a longer lag period. To explore the duration of the response of the marker genes to γ irradiation, we next looked at longer postirradiation incubation times. Most of the markers were maximally induced at 24 h after irradiation, followed by a gradual decline in the magnitude of response (Fig. 3). *PCNA* levels, however, appeared to increase slightly between 24 and 48 h after exposure. The transcript for *IFNG* p10, while not significantly elevated at 24 h after irradiation, was significantly elevated after 48 and 72 h. The average elevation of all the markers examined remained significantly above background levels 72 h after radiation exposure, although with greater variability among donors.

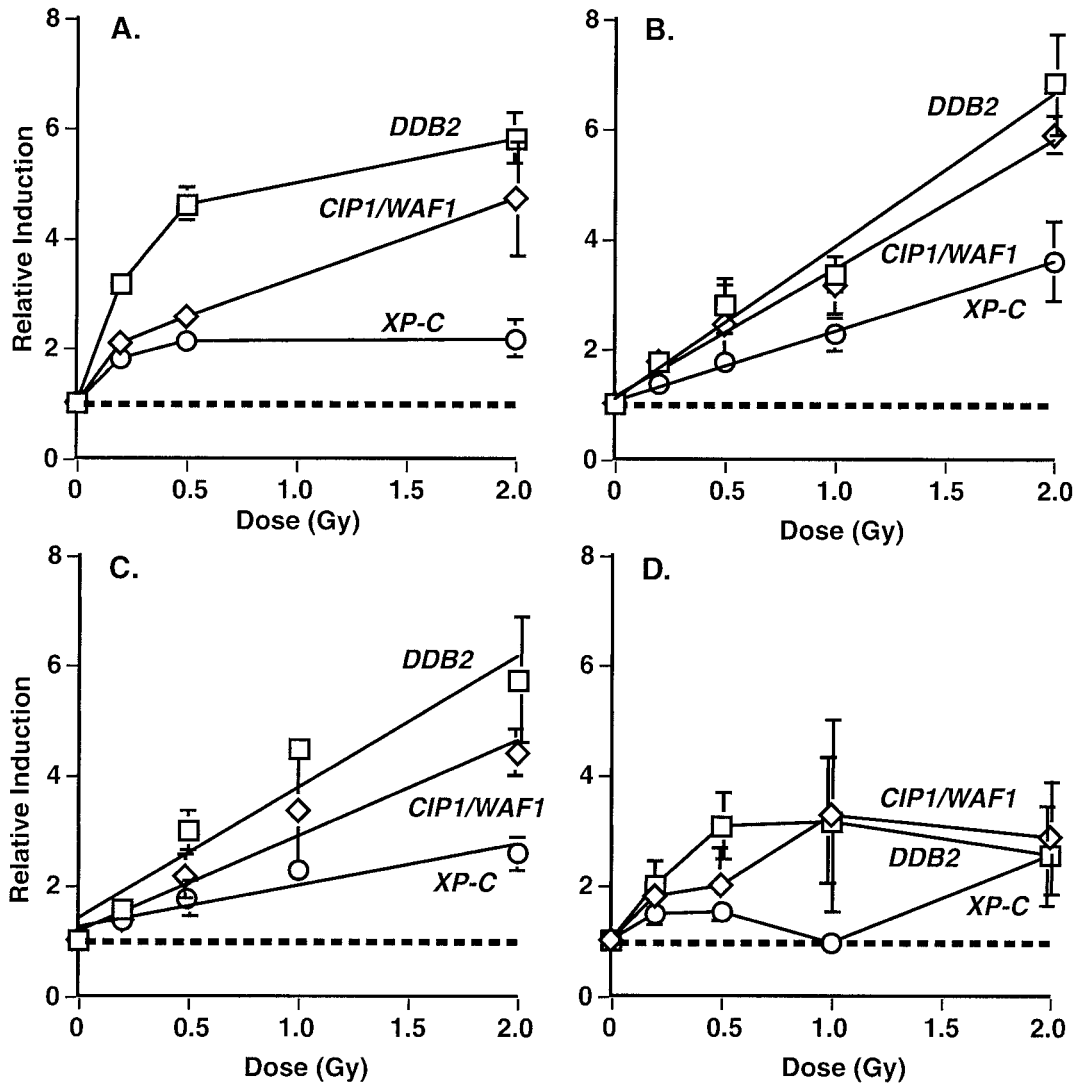


FIG. 4. Dose–response relationship for the induction of *DDB2* (□), *CDKN1A* (CIP1/WAF1) (◇), and *XPC* (XP-C) (●) in human PBL. PBL were irradiated *ex vivo* with graded doses of γ rays and RNA was harvested after (panel A) 4, (panel B) 24, (panel C) 48 or (panel D) 72 h of incubation. Points are the mean of relative expression in three or more independent donors, and error bars are standard errors of the mean. Where error bars are not visible, they are smaller than the height of the points on the graph. The dashed lines indicate the level of expression in unirradiated control cultures.

Finally, we investigated the dose–response relationship for the induction of *CDKN1A*, *DDB2* and *XPC* in PBL at various times after irradiation. All three of these genes were reproducibly induced in PBL from different donors by doses of γ rays as low as 20 cGy. Gene induction by doses of 50 cGy or less appeared to reach their maxima by 4 h after irradiation (Fig. 4A). By 24 (Fig. 4B) and 48 h (Fig. 4C), induction by the higher doses had peaked, with all three genes following a linear dose–response relationship. For all three genes, the slope of the induction curves was somewhat shallower after 48 h than at 24 h. By 72 h after irradiation, the dose–response relationship is no longer evident, due largely to the introduction of much wider variability in the responses between different donors (Fig. 4D). In fact, in some donors, there was no longer a measurable increase of all three transcripts above the levels in the untreated controls, although all donors did show significant

elevation of one or more transcripts at this late time. This variability may be due to the decay of the DNA damage signal, or it may reflect an approach to the time limit for maintenance of healthy quiescent PBL in culture. Daily cell counts were relatively stable during the course of the experiments, however, and showed little decline in overall cell number. The percentage of nonviable cells in untreated cultures as measured by trypan blue dye exclusion averaged around 1%, and the percentage of apoptotic cells as determined by DAPI staining was similar. Only a slight increase in these two measures of cell viability (still less than 5% of the population) was noted up to 3 days after 2 Gy γ rays. This indicates that the viability of the lymphocyte cultures did not change dramatically during the course of these experiments.

The prominent up-regulation of *DDB2* and *XPC*, two components of the nucleotide excision repair pathway,

gives further support for the recently reported link between UV and ionizing radiation repair pathways (12). However, in view of the recent demonstration of the dependence of radiation induction of *DDB2* on wild-type *TP53* status (13), and the well-defined role of *TP53* in regulation of *CDKN1A*, *PCNA* and *CCNG1*, the gene inductions observed in PBL may simply reflect a general *TP53* activation response. We have since observed a strong correlation between wild-type *TP53* status and ionizing radiation induction of *XPC* in human tumor cell lines (manuscript in preparation). This and related studies may implicate the activation of *TP53* as a principal determinant of gene induction responses to ionizing radiation in human PBL.

It should also be noted that the radiation doses used in these experiments are in a range relevant to accidental human exposures. For instance, during the Chernobyl reactor accident, over 200 emergency workers and staff received doses greater than 1 Gy, while the range of exposures to workers and the surrounding population was estimated at the time to be between 0.1 and 13 Gy or more (14). Chronic exposures due to dumping of radioactive waste in the Techa River resulted in accumulated doses to the nearby population between 0.05 and 2.3 Gy and 940 diagnosed cases of chronic radiation sickness (15). In comparison, the LD_{50/60} (the radiation dose that will be lethal to 50% of exposed individuals within 60 days) for humans is around 4 Gy and may be increased to as much as 8 Gy with appropriate treatment (16). These doses are much in excess of those used in the present study, where the highest dose was 2 Gy, corresponding to a standard radiotherapy fraction as used in cancer treatment.

After accidental exposures, increases in chromosome aberrations have been detected in people receiving doses above 0.5 to 1 Gy, and electron spin resonance of dental enamel can be sensitive to exposures as low as 0.1 Gy (13). However, neither of these techniques has the potential for high-throughput assay development for large-scale population monitoring that gene expression assays have. In addition, the electron spin resonance technique, though considered a sensitive radiation biodosimeter, requires extraction of a tooth, and thus may be considered overly invasive in many populations.

We have demonstrated the application of cDNA microarray analysis to discover potential markers of radiation exposure in human PBL, and we have shown the reproducible induction of several of these markers in *ex vivo*-irradiated PBL from multiple independent donors. The fact that the expression levels of the genes examined in unirradiated PBL varied only slightly between donors, and that relative induction of these genes by graded doses of *ex vivo* irradiation was similar in all donors, supports their potential usefulness as markers of radiation exposure. It may be possible to establish normal ranges for expression levels of these genes to distinguish irradiated individuals, whose ex-

pression levels of these genes would fall outside the normal range. An approach evaluating a set of genes rather than a single transcript would likely produce more accurate estimates of exposures.

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