

Gene-Expression Biomarkers for Application to High-Throughput Radiation Biodosimetry

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ABSTRACT

Molecular biodosimetry tools are a valuable asset for life-saving medical triage and patient management following a radiological or nuclear disaster. Even with the delayed onset of symptoms, sometimes several days after exposure, gene-expression biomarkers can identify these exposed individuals very early after exposure, allowing for prompt medical intervention. This early assessment of a radiation dose after exposure would enhance the operational commander's situational awareness of the radiation exposure status of deployed units and increase the prospect of reduced morbidity and mortality through early medical intervention. Candidate gene targets were selected from microarray studies of ex vivo-irradiated human peripheral blood lymphocytes and measured using a quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) assay in a human whole blood model at 24 and 48 hr post-irradiation (0.2 to 5.0 Gy 60Co-gamma rays at 0.1 Gy/minute). A multi-target QRT-PCR technique was optimized, an inter-individual variation study of an ex vivo response was expanded to include a larger cohort of healthy donors, and a collaborative study was conducted involving the in vivo response of cancer patients undergoing total body irradiation treatment. These validation studies illustrated that gamma radiation causes quantifiable and reproducible gene-expression changes in the radiation biomarker targets CDKN1A, BAX, GADD45A, and DDB2 over several days across a broad dose range in both in vivo and ex vivo models. However, further validation studies are required before these gene targets can be used as molecular biodosimeters. Once validated, the gene-expression signatures of several sentinel biomarkers would provide an early dose estimate that would then be used when making decisions involving personnel operational capabilities and/or clinical therapy. In addition, we are implementing validated protocols for automation to create high-throughput clinical and deployable PCR-based diagnostic platforms. With the increased throughput, the use of the Armed Forces Radiobiology Research Institute's Biodosimetry Assessment Tool (BAT), and a multi-parameter biodosimetry diagnostic approach, we can determine an individual's exposure dose in a mass-casualty scenario, which would aid decision-making for medical triage (greater than 10 cGy).

1.0 INTRODUCTION

Medical management of radiation casualties (e.g., triage, emergency care, and definitive care) must rely on clinical signs and symptoms for dose estimates [Browne 1990, AFRRRI 2003]. Typically, physical dosimetry and cytogenetics are used to identify persons exposed to less than 1 Gray in emergency accident response and

retrospective dose assessment [Zoetelief 1990, Muller 1991]. However, rapid, reliable and minimally invasive biodosimetry assays are needed to facilitate triage and case-management decisions. Both qualitative screening and quantitative dosimetry methods are needed. Sophisticated quantitative assays using proteomic and genomic technologies are in the early phases of research and development. Further research is required before these biomarker assays can be used to make clinical correlations between ionizing radiation dose exposures and changes in expression of sentinel biomarkers. Potential indicators of radiation exposure include molecular gene expression [Miller 1998, Amundson 1999, Blakely 2003b] and protein biomarkers [Blakely 2003a, Miller 1998] from whole blood. Other tissue and fluid sources of cellular RNA and protein could include cells found in hair follicles, buccal smears, urine, semen, and feces. Once the molecular biomarkers are validated for specificity to radiation injury, the rapid high-throughput and multiplex detection methods also must be validated for sufficient assay sensitivity and reproducibility. Moreover, gene-expression biomarkers may prove to be informative about an individual's genetic susceptibility to radiation exposures.

These biomarkers are required because, in many scenarios, personal physical dosimeters are not worn on the body. When correctly worn, these dosimeters have the ability to accurately assess radiation exposure over a suitable broad dose range. Personal dosimeters measure the radiation dose to the dosimeter only, and not to the potentially exposed individual. Exposure scenarios to potentially high levels of penetrating ionizing radiation can involve non-uniform fields with exposures of the whole body, a significant portion of the body, or a small, localized part of the body. Personal dosimeters could record a very low absorbed dose, while a substantial partial-body dose is received, making it difficult to establish an accurate radiation dose assessment. Radiation exposures are likely to be complex, and several parameters will influence dose estimation. There is no single assay that can work well in all potential scenarios.

The requirement for a multi-parametric approach to biodosimetry has led to the search for other possible bio-indicators of radiation injury. We are developing a multifaceted and integrated biodosimetry system at AFRRRI that will address fully the need for triage to differentiate between the exposed and the "concerned public," and to support clinical management of radiation accident victims [Blakely 2002a]. Our long-range goal is to develop and integrate a battery of validated radiation bioassays to equip medical personnel with diagnostic information relevant to the medical management of human radiation casualties. Our specific objectives are to: (a) establish definitive, rapid, high-throughput clinical bioassays for radiation dose assessments, (b) develop complementary triage-type radiation dose-assessment bioassays, such as molecular biology-based, forward-deployable diagnostic platforms, and (c) transfer the Biodosimetry Assessment Tool (BAT) software program and other complimentary tools to healthcare professionals to facilitate the collection, integration, and archiving of relevant clinical signs, symptoms, physical and other biological dosimetry information [Salter 2004, Sine 2000].

We hope to be able to validate a group of informative gene-expression biomarkers from the plethora of gene-expression targets identified by microarray analysis (Figure 1A). It is now well established that ionizing radiation induces a cellular response of a complex pattern of gene-expression changes to injury by ionizing radiation [Amundson 2000, 2001]. Specific patterns of radiation-induced gene-expression changes can be analyzed using bioinformatics from micro-array (genomic chip) technology (Figure 1B) to interpret and analyze "macro-scale" biomarker data (Figure 1C). Emerging data indicate signature patterns of radiation-induced gene-expression changes in different cellular pathways. These preliminary studies likely will lead to identification of sentinel molecular targets to serve as biomarkers for human radiation exposure, treatment therapies, and identification of the underlying genetic determinants that are responsible for the differences in radiation sensitivities within populations. Novel biomarker assays must be standardized, validated, and relatively easy to measure. Quantitative reverse-transcription polymerase chain reaction (QRT-PCR) provides a rapid, cost effective, reproducible platform for model system and gene target validation studies (Figure 1D). In the near fu-

ture, microarray and PCR technologies will enable large-scale, low-cost analysis systems with the use of automated workstations for extracting nucleic acid isolation and performing DNA amplification, hybridization, and detection.

Currently, microarray and PCR technologies are being applied to available model systems such as irradiated *in vitro* cell culture, *ex vivo* irradiated whole blood, and *in vivo* models such as spleen cells from irradiated mice or blood from radiotherapy patients (Figure 1A). Emphasis now should be placed on validation studies and on comparison of candidate biomarkers in these surrogate models to the molecular and cellular changes that would occur in irradiated subjects (Figure 1A–D). Radiation-induced gene-expression changes can be evaluated with QRT-PCR assays to validate sentinel biomarkers (Figure C–D) [Blakely 2001, Grace 2002, Amundson 2004]. Studies with QRT-PCR assay may be smaller, shorter, and cheaper than microarray studies. Several gene-expression biomarkers may be required to accurately assess radiation dose or injury because of inter-individual genetic variations. Further studies are needed to determine the number of radiation-responsive gene targets that are required to enable correct predictions of radiation dose and injury in a large population. In order to accomplish these studies, regulatory approved, high-throughput platforms must be validated.

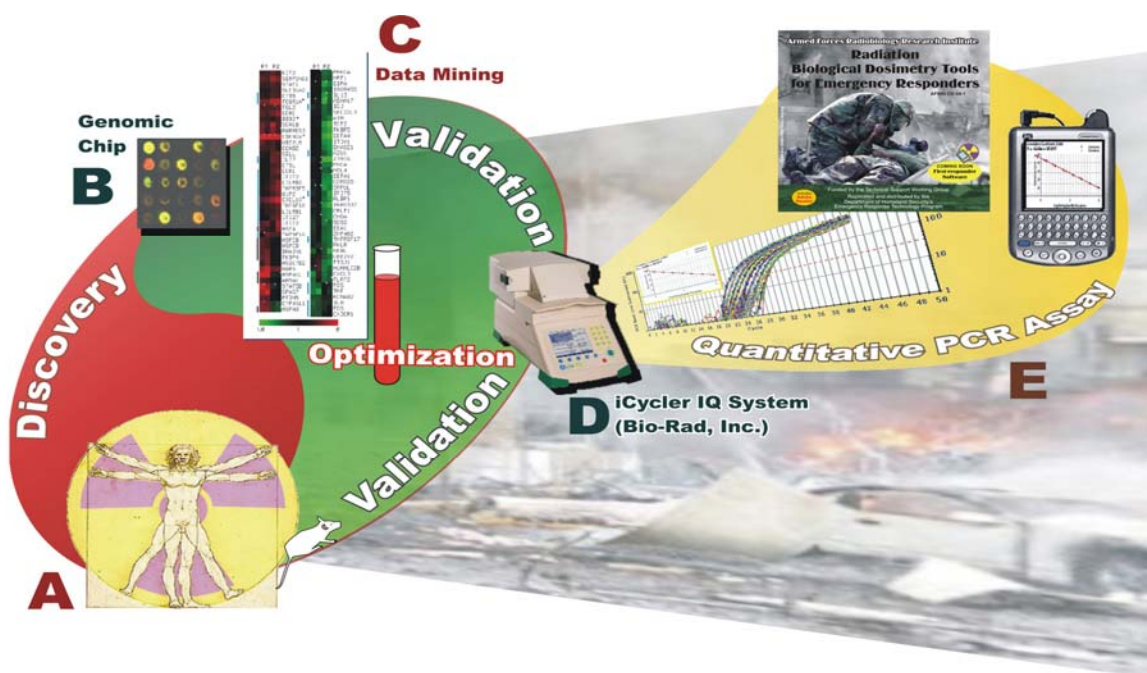


Figure 1: Candidate biomarker discovery to biosimulator assay assimilation. Genetic model validation (A–D) and target validation (C–D) are part of the extensive validation studies for the gene-expression targets. (A) Model system resources (e.g., *in vitro* cell cultures, *ex vivo* irradiated human whole blood, *in vivo* radiotherapy patient models, or animal models, etc.) are needed to ensure the accuracy and precision of novel biomarker assays. Easily accessible tissues like blood, buccal, or hair follicles are used for gene target discovery and validation studies. (B) Actual microarray data [Amundson 2004]. (C) Data mining strategies are used to interpret and analyze biomarker data obtained from gene-expression patterns. (D) QRT-PCR multiplex platform for validation studies. (E) Validated sentinel biomarker groupings can be used to meet end-user requirements for triage and definitive diagnostic information by development of a clinical and forward-field QRT-PCR assay. PCR data can be downloaded to hand-held or laptop PC computer devices.

2.0 FROM CANDIDATE GENE-EXPRESSION BIOMARKERS TO REGULATORY APPROVED BIODOSIMETER DEVICES

The Biomarkers Definitions Working Group (BDWG) of the National Institutes of Health [Biomarker Definitions Working Group 2001] defines a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.” Biomarkers that are used to predict radiation sensitivity, radiation dose, or injury are referred to as surrogate endpoints. A surrogate endpoint, such as a gene signature, may be used to predict radiation dose, injury, or lack of injury based on quantitative measurements in models. Radiation biomarker validation studies must show substantial evidence of a linkage to ionizing radiation exposures, and also should characterize the biomarker’s response to other confounders that might affect the surrogate endpoint, such as exposures to biological or chemical agents. Biomarkers must be validated by documenting quantifiable statistical accuracy, sensitivity, specificity, and reproducibility. We have begun extensive studies to identify, evaluate, and validate molecular biomarkers that may provide diagnostic information for acute and prior radiation exposures. These studies contribute to understanding the function of these candidate gene-expression biomarkers in irradiated and non-irradiated populations, as well as to their sensitivity, specificity (i.e., chemical, biological, radiological), rates of assay false positives and negatives, alone and in cases of mixed exposures. These surrogate endpoints also must be characterized for radiation quality, dose rate, and whole or partial body exposures. Only then will these validated biomarker assays complement conventional chromosome-aberration assays for early biological dose assessments. To meet this formidable task, we have implemented biodosimetry validation studies using high-throughput robotic and real-time PCR technologies.

2.1 Ideal Characteristics for Gene-Expression Biomarkers of Radiation Response

- **Specific**
 - Non-responsive to other insults (e.g., chemical, or biological agents)
- **Sensitive**
 - Low baseline to detect up-regulated genes*
- **Reliable**
 - Low inter-individual variation
- **Robust**
 - High signal to noise for radio-response
- **Predictive**
 - Long half-life with dose and time-dependent changes post-exposure
 - Meaningful radio-response with different radiation qualities and dose rates
- **Noninvasive**
 - Blood
 - Semen
 - Buccal
 - Hair follicles
 - Urine
 - Fecal

*Current diagnostic technology makes it easier to develop and optimize methods and assay controls for monitoring *up-regulation* of gene products than for monitoring *down-regulation* of these products. It is more difficult to distinguish a failed assay for a low abundance target than a “true” negative result. Thus a low baseline value in unexposed individuals is optimal for studying a radiation response.

3.0 SEMINAL STUDIES OF RADIATION GENE-EXPRESSION BIOMARKERS

Although the human peripheral blood lymphocyte-dicentric assay is the “gold standard” for dose assessment,

complementary methodologies that give immediate information are needed for both forward-deployable and clinical biodosimetry capability. The U.S. capability in this area is inadequate for a national emergency or military operation. However, the validity of the fluorescent RT-PCR assay has been developed, and use of kinetic RT-PCR assays for quantification of gene-expression biomarkers have been established for many paradigms, including radiation biodosimetry, that are published in peer-reviewed literature.

In general, the modulation of gene-expression radioresponse is thought to be related to the cellular capacity to survive injury. Recent studies have demonstrated that the early response genes *c-jun*, *Egr-1*, *c-fos*, and NF kappa B are induced following exposure of mammalian cells to ionizing radiation [Weichselbaum 1994]. The products of these early response genes regulate downstream genes that are important in the adaptation of cells and tissues to radiation-induced stress. Potential downstream targets include cytokine and growth-factor genes as well as deoxyribonucleic-acid (DNA) repair genes. Early response gene products also may regulate cell cycle progression following ionizing irradiation. Signal transduction pathways that allow cells to adapt to radiation may provide gene-expression biomarkers for normal responses and patient treatments to radiotherapy [Weichselbaum 1994].

Proof-of-principle for a candidate nucleic acid biomarker (i.e., gene-expression target) responsive to ionizing radiation was identified at AFRRI using an *in vitro* model system of human peripheral blood lymphocytes. A dose-dependent elevation in *Haras* gene-expression levels was demonstrated using Northern blot analysis at 17 hr after exposure to 250-kVp X rays (25-100 cGy; 1 Gy/min) [Blakely 2002a, Miller 2002]. Inter-individual variation did not appear to be a confounder either *in vitro* with a healthy donor human peripheral blood leukocyte (HPBL) model or *in vivo* using a murine model [Blakely 2002b, 2003a, Miller 2002]. These studies support the proof-of-concept for use of gene-expression biomarkers as radiation biodosimeters. However, further studies to identify and optimize analysis systems, and to validate gene targets for necessary regulatory approvals are necessary.

4.0 DISCOVERY AND VALIDATION

4.1 Biological Models for Discovery and Validation of Gene-Expression Biomarkers

Data mining (Figure 1C) by microarray technology is the process of analyzing complex data from different test groups (such as non-irradiated or irradiated cell cultures, whole blood radiation models, or irradiated animals) identified on microarray or genome chips, and summarizing up-regulated (red-coded fluorescence) and down-regulated (green-coded fluorescence) genes from various tissue biopsy models with a number of different analytical tools by bioinformatics. These data can be analyzed from many different dimensions or viewpoints, categorized, and summarized so that possible relationships can be identified. Technically, gene-expression data mining is the process of correlating gene-expression patterns among dozens of fields in large relational databases from various cell culture and animal model systems with different degrees of predictable dose-response and relevance to human injury.

The fact that gene-expression changes can be found *in vitro* with a cell line or *ex vivo* with a whole blood irradiation model does not mean that the same change can be found *in vivo*. In our studies at AFRRI, microarray data from human peripheral blood lymphocytes (HPBLs) have proven to be the most useful model for validation studies by QRT-PCR assay in the human whole blood *ex vivo* irradiation model [Grace 2002, 2003]. The choice of a species for *in vivo* validation studies of gene-expression biomarkers must take into consideration the genetic similarity of the choice of species to humans, genetic diversity within the species, breeding cycles, housing costs, and personnel requirements. The mouse is easy to work with, genetically elucidated, and has a

similar genome size and structure to the human counterpart. Although similar, the mouse genome is about 14% smaller than the human genome, the difference probably derived from an increased genomic deletion rate in the mouse. This difference could be a confounder when using specific surrogate endpoints in otherwise conserved gene pathways. Among remaining species, monkeys are, in general, more similar to humans than any other species except the great apes, and apes are so endangered and expensive to handle that their use is virtually eliminated, except in rare cases. In some studies, the monkey could be the most suitable model for human target validation. Other practical larger animal models include rats, sheep, rabbits, and pigs.

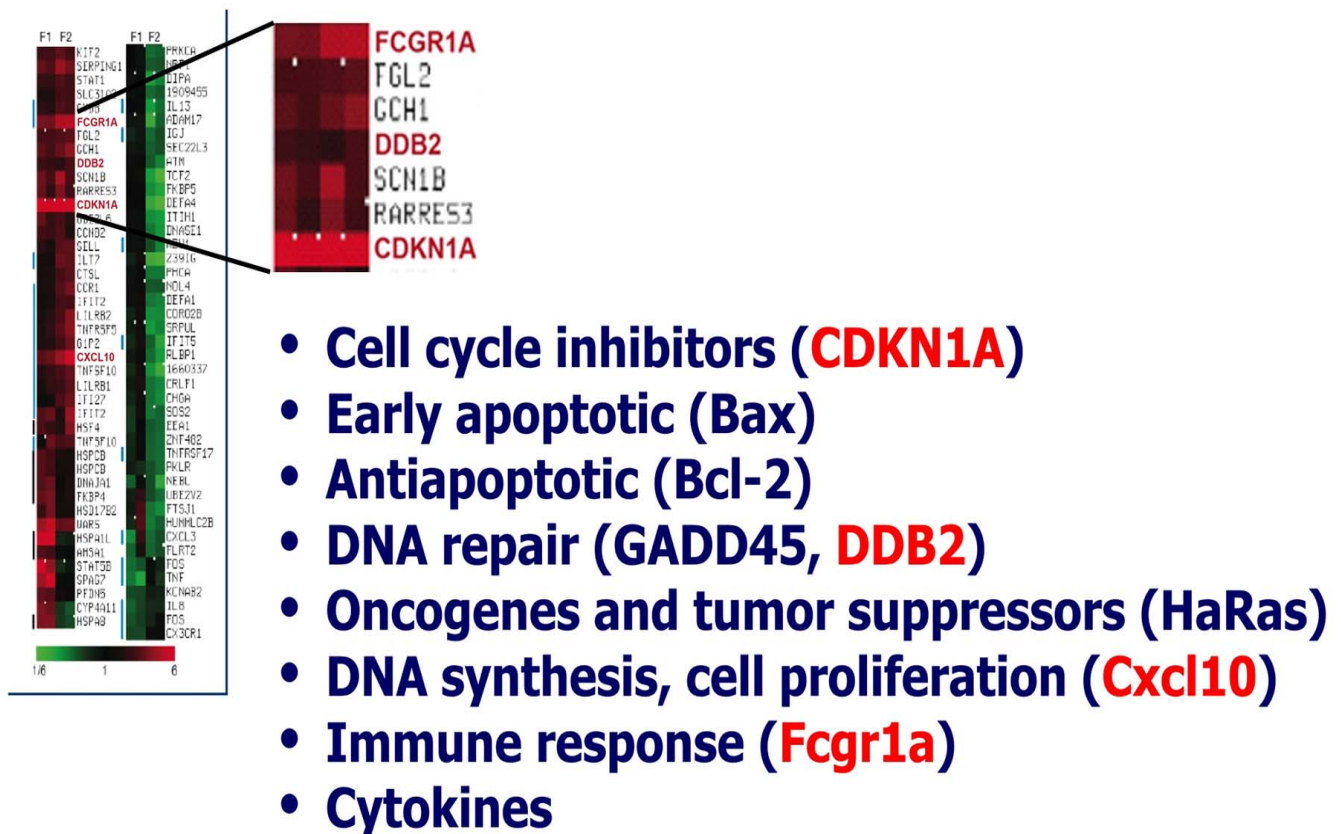


Figure 2: Left panel: Hierarchical clustering of genes significantly up- or down-regulated in 2 or more micro-array hybridizations with minimum red or green intensity of 500 and minimum mean quality of 0.5. The color scale at the bottom of the figure shows the expression after treatment relative to the pre-exposure control. Genes studied by QRT-PCR are indicated in red. [Amundson 2004] **Right panel:** Candidate gene targets for biological validation are taken from microarray data and validated by QRT-PCR assay using prior-literature searches involving known cell-cycle pathways.

Once an appropriate model is chosen, functional genomic approaches (Figure 1A), such as microarray hybridization analysis, provide state-of-the-art methods for the discovery of potentially informative radiation-exposure gene-expression profiles. Once validated, a set of biomarker genes could be developed into a simple, rapid, and reproducible assay using a quadruplex QRT-PCR multiplex platform (Figure 1D) [Grace 2003]. Initial microarray studies of *ex vivo*-irradiated HPBL (Figure 1B, C) supported such an approach [Amundson 2003]. Dose-response relationships were validated by QRT-PCR assay for a number of genes [Grace 2003] in

whole blood with little variability among healthy donors. These studies confirmed the feasibility of multiplex QRT-PCR assay biodosimetry in an assessable tissue such as whole blood. In order to develop useful gene-expression biomarkers, gene-expression changes that occur in response to ionizing radiation must be validated by *in vivo* models. Human radiotherapy patients provide a convenient and relevant test population for supporting *in vivo* validation studies (Figure 2, left panel). In collaboration with the Fornace laboratory (NIH, Bethesda MD), we were the first to publish *in vivo* induction of expression of the *CDKN1A*, *GADD45A* and *DDB2* genes along with the identification of additional potential *in vivo* exposure marker genes in a set of patients undergoing total body irradiation (TBI) in preparation for allogeneic or autologous hematopoietic stem cell transplantation (Figure 2, right). The information gathered in such studies also may contribute to a future increased mechanistic understanding of individual response to radiation therapy [Amundson 2004].

4.3 Analytical Platforms for Measurement

Recent rapid advances in nucleic acid biomarker analysis systems have realized this possibility of forward-field, individual dose assessment. Rapid fluorogenic quantitative PCR assay systems currently are available commercially off-the-shelf for research and clinical laboratories. Military operations already rely on molecular biology analysis platforms for both forward-field and clinical evaluations. The ABI Prism 7700 from Applied Biosystems (Foster City, CA, USA) was the first platform to contain a built-in thermal cycler with 96 well positions able to detect laser fluorescence between 500nm and 660nm. One plate-run takes about 2 hr to complete and can be viewed immediately after the finish of the amplification run. An example of a four-color detection and cost-efficient unit is the Bio-Rad iCycler iQ™. It provides a 96-well format with optical caps that can be set up with pre-developed reagent and assay-validation plates of practical use to diagnostic applications. A broad-spectrum tungsten light source and multiple filters permit a wide range of excitation and emission between 400-700 nm, as well as the multiplexing of up to four different reporter fluorophores. On-line display allows visual confirmation of the PCR amplification in progress. The output provides a useful advantage of instantaneous visual representation of the amplification process for each sample. This feature is helpful particularly in a military field laboratory or a point-of-care medical facility where QPCR and QRT-PCR assays are most challenging. Troubleshooting steps can be implemented without delay by examination of the real-time data for signal detection of endogenous and exogenous control targets. Optimized and validated protocols for analytical systems were developed for rapid measurement of radiation-responsive gene-expression biomarkers on both platforms at AFRRRI [Grace 2002, 2003].

4.3.1 Quadruplex, Quantitative Reverse Transcription-PCR Assay

We were the first laboratory to optimize and validate a four-target multiplex QRT-PCR assay methodology for simultaneous quantitative detection of three radiation-specific gene-expression biomarkers and an internal control using RNA isolated from whole blood [Grace 2003]. We expect to be able to estimate dose within a 0.5 Gy window by assessing 6–12 sentinel biomarkers in *ex vivo* and *in vivo* model systems. These QRT-PCR assays have been demonstrated in our laboratory to be specific, reproducible, and rapid [Grace 2002, 2003]. We have demonstrated that whole blood samples readily provide access to unique gene profile signatures that are capable of differentiating the severity of possible exposures to the host. We have completed studies to evaluate the efficacy of these endpoints to assess acute radiation exposure in *ex vivo*-irradiated human whole blood, and have begun studies using *in vivo* murine and cancer patient model systems. On-going studies include inter-individual variation in dose-response using an *ex vivo* irradiation model by measuring radiation-response gene targets by multiplex QRT-PCR assays. Total RNAs were isolated from whole blood in three healthy subjects for a broad dose range (0.25-3 Gy) and from eighteen healthy subjects for 0.25 and 2.5 Gy doses. The fitted function of data from the larger cohort in log scale (0.25 and 2.5 Gy) was chosen for statisti-

cal analysis of prediction of dose from a calibration curve.

We measured several potential radiation-responsive gene-expression biomarkers, (i.e., *GADD45*, *DDB-2*, *BAX*, and *CDKN1A*) at 24 and 48 hr post-irradiation [Grace in preparation]. Figure 3 shows an example of data from two gene targets, *GADD45* and *DDB2*, at 24 hr post-irradiation using the whole blood irradiation model.

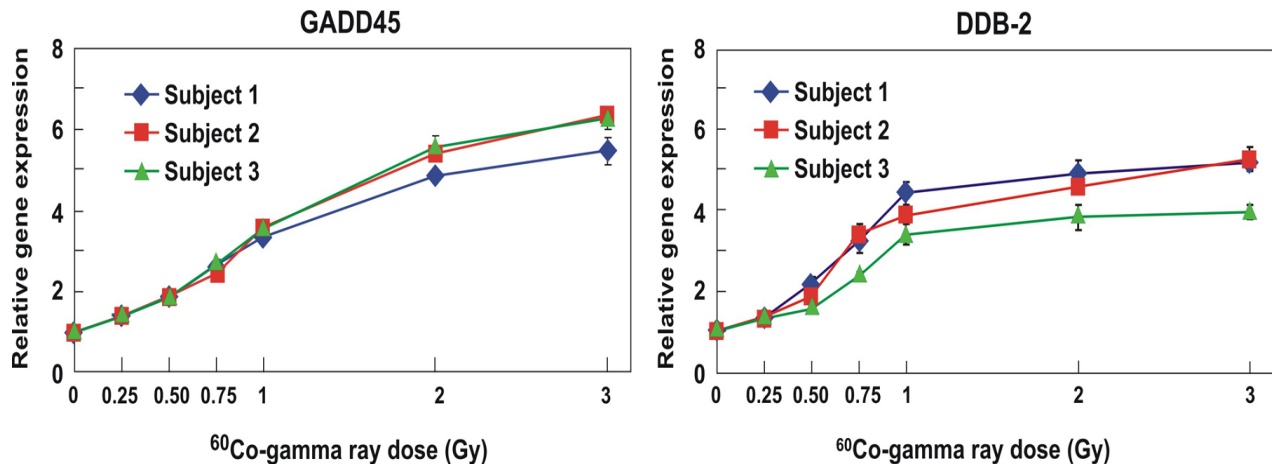


Figure 3: Relative gene-expression dose response using whole human blood model at 24 hours (Left panel: *GADD45*; right panel: *DDB2* gene targets) Relative quantification (RQ): Gene target values were normalized to 18S ribosomal subunit with the formula $\Delta C_T = \text{target } C_T - \text{internal standard } C_T$. Relative gene-expression levels after irradiation were obtained from comparison with the pre-irradiation control C_T used as the reference standard.

In collaboration with the Fornace laboratory at NIH, we were the first to publish a study in radiotherapy patients receiving total-body irradiation treatments [Amundson 2004]. In these studies, real-time RT-PCR based on iCycler IQ technology validated DNA array results in radiotherapy patients for five gene targets (Table 1). QRT-PCR assay proved to be quantitative, rapid, and required 1000-fold less RNA than microarray assay. Qualitatively, both microarray and real-time RT-PCR analyses gave comparable results for five genes, i.e., relative gene-expression levels were qualitatively in agreement by both techniques (Table 1). These data, however, also demonstrate the limitations of microarray sensitivity and specificity when compared to the quantitative PCR assay (Figure 4). Real-time PCR data are quantitative because of specific primer and probe design for DNA amplification, universal hot-start PCR methodology, and multi-target detection with an internal standard [Grace 2003]. Microarray experiments are more prone to a number of different errors, hybridization inhibitors, and assay variability as evidenced by typical data variability (Table 1), as shown in Figure 4. Perfect agreement would predict data symbols shown in Figure 4 to fall on a line with a slope equal 1.0, which is approached by the data shown for two of the five gene-expression targets (*FCGR1A*, *CXCL10*). Due to the enhanced precision and accuracy, the QRT-PCR assay has greater potential for assessing dose and injury determinations in radiation biodosimetry applications.

Another limitation of microarray analysis is obtaining sufficient quantities of cellular total RNA from whole blood to do the microarray analysis after TBI treatments. This difficulty proved to be the case after a total dose of 3 Gy in a TBI patient (Table 1). Microarray data was only obtained before treatment and after the first two treatments of TBI. To further establish the *in vivo* radiation response of gene expression, we determined

gene expression by QRT-PCR assay beyond the range of the obtainable microarray data. We followed the same patient's response through six fractions of TBI treatment for eight genes, *CXCL10* and *CDKN1A* (Figure 5A), *DDB2*, *FCGR1A*, and *GADD45A* (Figure 5B), and *Bax* and *Bcl-2* (Figure 5C). The QRT-PCR assay data were extended for a total three-day course of TBI for a 9-Gy accumulated dose.

While *FCGR1A* expression leveled off above 3-Gy cumulative dose (Figure 5A), expression of *DDB2*, *CDKN1A* and *CXCL10* (Figure 5A, B) [Amundson 2004] and preliminary data for *BAX* and *BCL-2* targets (Figure 5C) continued to increase with accumulating dose through the course of radiation treatment. In contrast, *GADD45A*, which appeared unresponsive in the microarray data through the first two fractions, actually decreased following the later TBI fractions (Table 1, Figure 5B). It should be noted that the initial basal level of *GADD45A* in this patient was extremely high and within the range of the post-induction levels seen among the other patients.

Gene	Dose 1		Dose 2	
	Array ¹	qPCR ²	Array ¹	qPCR ²
GADD45A	0.95	1.17	1.8	0.69
CDKN1A	5.4	26.0	7.5	24.0
DDB2	2.0	4.3	2.0	2.5
FCGR1A	2.9	3.1	4.4	4.3
CXCL10	2.5	9.5	5.5	16.3

¹Mean of two determinations
²Mean of 9 determinations (6 for DDB2).

Table 1: Qualitative comparison of results for five gene targets by microarray and real-time RT-PCR assay. Gene expression was determined in a radiation therapy patient after the first two TBI treatments (1.5 Gy). Microarray data were derived from analysis of two independent determinations of array data as specified [Amundson 2004]. Real-time PCR data were obtained with the Comparative C_t method as previously described [Grace 2003]. Two or three independent real-time RT-PCR experiments for each target were carried out.

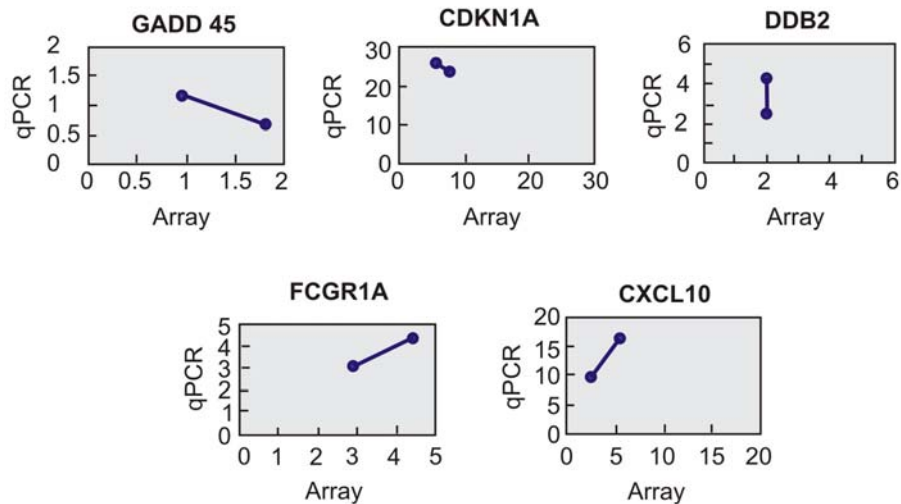


Figure 4: Relative gene-expression measurements by QRT-PCR assay (Y-axis) compared to measurements by microarray analysis (X-axis) from Table 2. Comparison of relative induction levels by quantitative PCR assay (Y-axis) and microarray data (X-axis) does not approach theoretical values.

To further establish the radiation response of gene expression, we determined gene expression by real-time RT-PCR in six randomly selected post-TBI (1.5 Gy) treatment patient samples for seven genes (*CXCL10*, *CDKN1A*, *DDB2*, *FCGR1A*, *GADD45A*, *BAX*, and *BCL-2*). We determined the fold-change in gene expression by RT-PCR in six paired patient samples (pre- and post-TBI treatment). All of the genes examined in Table 1 showed variability in responses between different cancer patients, although *CDKN1A* was induced in all patients [Amundson 2004]. However, the pro-apoptotic gene, *BAX*, was induced similarly in these six patients, and anti-apoptotic gene *BCL2* also was expressed in similar levels (Figure 5 D). These two genes are among our promising exposure biomarkers from *ex vivo* radiation response work. Despite some apparent heterogeneity of individual responses to *in vivo* irradiation, they remain among the best of the tested candidates.

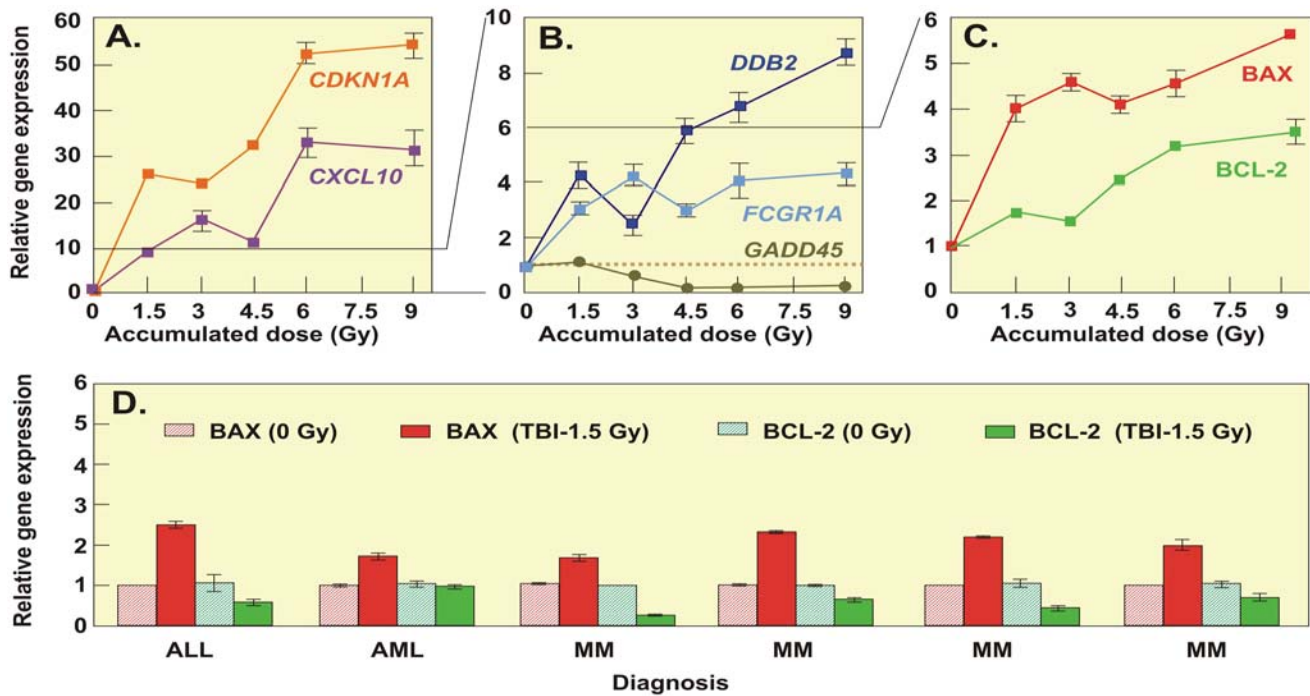


Figure 5: Measurements of relative gene-expression changes by QRT-PCR in various targets using samples derived from radiation-therapy patients. Panels A, B, and C: Response through the course of TBI treatment of a non-Hodgkin’s lymphoma patient [Amundson 2004]. The dashed line shown in panel B represents gene-expression levels (relative expression value of 1) prior to the start of TBI. Bars are the mean of 2 or 3 independent triplicate PCR reactions; error bars are standard error. The scale has been interrupted by a black bar on the Y-axis to illustrate the maximum induction levels for each graph. **Panel D:** Unpublished preliminary data showing relative induction of the pro-apoptotic gene *BAX* and the anti-apoptotic gene *BCL-2*, before treatment (0 Gy) and six hours after the first 1.5-Gy fraction of TBI in 6 random patients diagnosed with either acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), or multiple myeloma (MM). Bars are the mean of 2 or 3 independent triplicate PCR reactions; error bars are standard error.

The observed variations in individual responses substantiates our prediction that single genes would not have great value alone as diagnostic biomarkers, but that the overall expression pattern of a group of sentinel genes would be more informative. The radiotherapy patient model contributes to *in vivo* validation of candidate

biomarkers as a “transitional” study that bridges the existing gap between laboratory experimentation and population-based epidemiology. Ultimately, validation studies likely will require the use of non-human primate or other suitable animal models and large epidemiological studies to assess exposure-dose and injury-dose relationships.

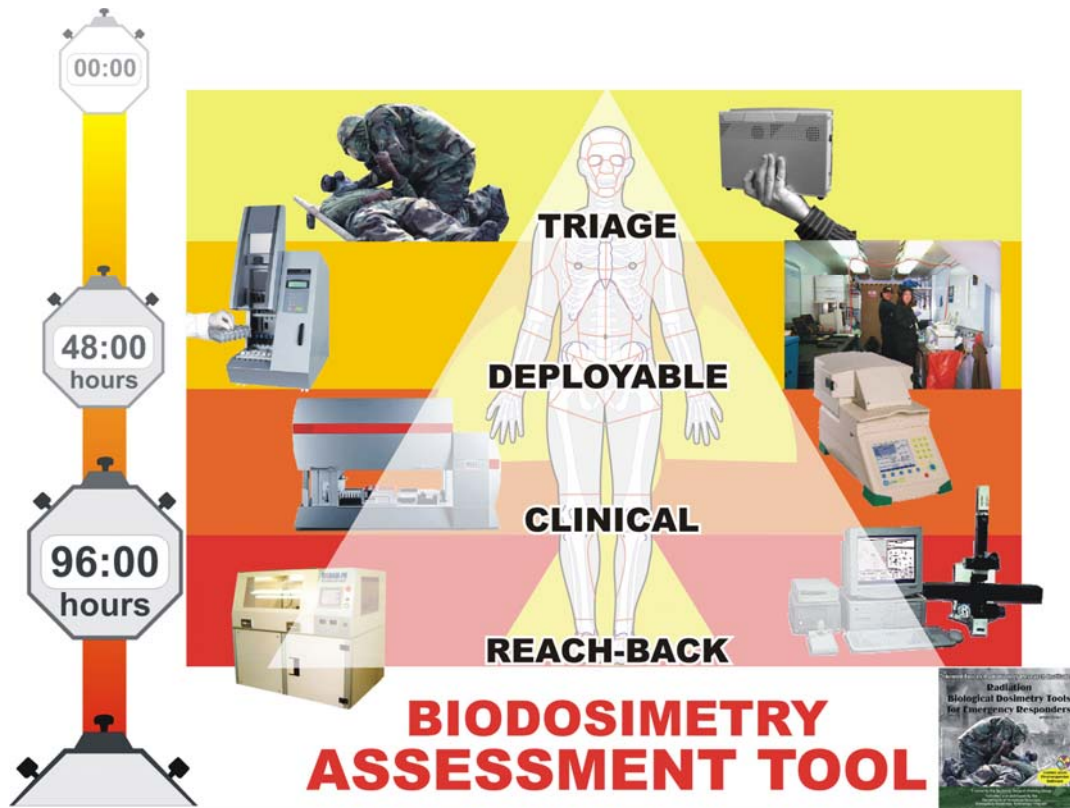


Figure 6: Schematic of timeline in hours using biodosimetry assays for a radiation incident (left) and hierarchy pyramid of equipment and software tools (right). First-pass triage assays, like protein strips and gene-expression hand-held devices, are inexpensive and small in size. These potential assays may distinguish dose—but could provide “yes and no” results with low false negative rates. Deployable assays include multiple target assays using hematology, protein, or gene expression and reduce the number of false negatives found in the first screening pass. The Qiagen EZ1 BioRobot (deployable, left) quickly isolates nucleic acids from whole blood samples using easy walk-away capability. The fieldable hematology laboratory (deployable, right) would be used for complete blood counts and lymphocyte-depletion kinetic studies for dose assessments. Clinical assays include hematology assays and precise dose estimates by QRT-PCR or Luminex protein-based assays that eliminate false positives, and distinguish dose, radiation-injury, whole or partial body exposures, and complex exposures (radiation, biological and/or agent, flu, HIV, etc.). The Qiagen BioRobot for fluid-handling system is illustrated (clinical, left) for high-throughput assays. The iCycler iQ PCR system is shown on the right for both deployable and clinical analysis. At the bottom of the pyramid, dose is confirmed by cytogenetics in a reach-back laboratory capacity. Blood samples are pipetted automatically in a sterile laminar-flow hood (reach-back, left) for culture setup. Use of an automated metaphase finder (reach-back, right) facilitates locating suitable metaphase spreads for scoring chromosome aberrations. AFRRRI’s Biodosimetry Assessment Tool software application permits recording of multiparameter indices relevant for radia-

tion dose assessment.

4.3.2 Forward-Field QRT-PCR Assay

Field-deployable systems for fluorogenic PCR assay currently are used in field military operation units for pathogen detection [O’Guinn 2004]. Hardened, small footprint instrumentation is required to equip forward deployable military laboratories and first responders. We have begun to test new products for transitioning RNA purification strategies and QRT-PCR-amplification formats to high-throughput robotics and hand-held or portable “hardened” devices for forward-field, point-of-care testing. These studies include the use of RNA stabilizers and systems with compatible chemistries to our current methodology for forward-field RNA isolations. We initiated preliminary studies by developing fieldable protocols for blood collection, stabilization of mRNA, RNA isolation, and QRT-PCR assay, and completed *ex vivo* time-dependent studies using the *ex vivo* radiation blood model-crossing formats on iCycler IQ for field use.

5.0 ASSIMILATION WITH BIODOSIMETRY ASSESSMENT TOOL SOFTWARE

5.1 Biodosimetry Assessment Tool Software

Validated gene-expression biomarkers can be used with the radiation-casualty-management software application Biodosimetry Assessment Tool (BAT). It permits entry of bioindicator data into dose predictions using either relative or absolute quantification values based on population studies or calibration curves. BAT was developed by the AFRRRI Biological Dosimetry Team. Version 0.75 has been released on the AFRRRI website (www.afrrri.usuhs.mil) for both military and civilian users [Salter 2004, Sine 2001]. Version 1.0 will be released at the same site in the near future. BAT equips healthcare providers with diagnostic information (clinical signs and symptoms, physical dosimetry, etc.) relevant to the management of human radiation casualties. Both versions 0.75 and 1.0 can provide immediate dose estimates in response to data entered regarding post-exposure lymphocyte counts and latency to vomiting. Once bioindicators such as gene-expression and protein biomarkers provide suitable predictive dose-responses and meet necessary validation criteria, we anticipate their inclusion in future updates to BAT.

Diagnostic biological dosimetry requires a multi-parametric approach and use of several standard assays, assimilating gene-expression biomarkers. BAT provides structured templates to record this physical dosimetry information that is useful in medically managing radiation casualties and helping triage individuals rapidly. An integrated and multifaceted approach that uses physical, hematological, cytogenetic, and molecular biodosimetry assays is essential for biodosimetry solutions for military personnel as well as civilian populations after a radiation disaster, whether deliberate attack or accident. BAT facilitates adequate and timely triage of radiation casualties and aids in effective clinical treatment decisions (Figure 6).

6.0 CONCLUSIONS

Currently, medical personnel mainly rely on clinical signs and symptoms of radiation exposure for early clinical treatment decisions. More accurate radiation-injury assessments, within hours to days post exposure, could result in more effective clinical management. Biomarkers of radiation exposure can be an important tool for triage of individuals in potentially overexposed populations following a radiological accident or “dirty bomb” incident. Thus, with appropriate medical intervention, the likelihood of survival can be increased significantly.

We are developing and validating simple, rapid, and automated biodosimetry tools for radiation dose assess-

ments that include high-throughput reference and forward-deployable assays with an emphasis on off-the-shelf, molecular-based diagnostic platforms in a multiparametric approach (Figure 6) [Prasanna 2004]. At the completion of this research, the contour of the exposure-to-gene-expression response relationship can be used to develop gene-expression batteries as screening tools for diagnostic radiation-exposure assessment. We hope to integrate the molecular biodosimetry data with BAT, to assist in medical management of radiation casualties. These validated assays will enable design concepts for future state-of-the-art biodosimeter devices by providing a validated effective group of biomarkers for radiation injury assessments.

The required validation studies are complex, involving technical, biological, and population validation studies. Proposed devices will be designed for the most probable scenarios of military and civilian radiation exposures and meet the requirements for first responders' triage tools and clinical assays. The assays that we described here will be used to validate future dosimeter systems that will be cheaper, non-invasive, faster, smaller, simpler, and more stable, with longer shelf life of reagents. Validation is the key to developing a hierarchy of triage assays that involve a multiparametric pyramid approach (Figure 6).

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