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Prediction of *In Vivo* Radiation Dose Status in Radiotherapy Patients using *Ex Vivo* and *In Vivo* Gene Expression Signatures

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Abstract

After a large-scale nuclear accident or an attack with an improvised nuclear device, rapid biodosimetry would be needed for triage. As a possible means to address this need, we previously defined a gene expression signature in human peripheral white blood cells irradiated *ex vivo* that predicts the level of radiation exposure with high accuracy. We now demonstrate this principle *in vivo* using blood from patients receiving total-body irradiation (TBI). Whole genome microarray analysis has identified genes responding significantly to *in vivo* radiation exposure in peripheral blood. A 3-nearest neighbor classifier built from the TBI patient data correctly predicted samples as exposed to 0, 1.25 or 3.75 Gy with 94% accuracy ($P < 0.001$) even when samples from healthy donor controls were included. The same samples were classified with 98% accuracy using a signature previously defined from *ex vivo* irradiation data. The samples could also be classified as exposed or not exposed with 100% accuracy. The demonstration that *ex vivo* irradiation is an appropriate model that can provide meaningful prediction of *in vivo* exposure levels, and that the signatures are robust across diverse disease states and independent sample sets, is an important advance in the application of gene expression for biodosimetry.

INTRODUCTION

Despite its many useful medical and industrial applications, ionizing radiation poses risks to both human health and national security. With the looming possibility of terrorists using an improvised nuclear device (IND) or radiological attack, the development of rapid biodosimetry methods is one of the highest priorities identified in the national plan for radiological/nuclear threat countermeasures (1). In a large-scale radiological event, radiation dose estimates would be essential for immediate medical triage and to evaluate later disease risks. Ideally, treatment should be initiated within 24–48 h after radiation exposure (2), which requires high-throughput assays for screening large populations. The gold standard for radiation biodosimetry, the dicentric assay, is currently impractical for mass triage (3,4) because it is too time consuming and requires highly trained personnel for scoring.

Current and emerging biodosimetry approaches have recently been reviewed (5) and include ongoing efforts to automate cytogenetic-based end points (6,7). Additional methods are also being developed, such as electron spin resonance (8,9), protein (10,11) and metabolomic

(12)-based methods. Gene expression signatures in peripheral white blood cells (WBC), which are relatively easily collected and highly sensitive in terms of early radiation-induced gene expression changes, provide another possible approach.

We previously suggested that gene expression profiles in human WBC can predict absorbed radiation dose within hours to days after exposure. In early studies we *ex vivo* irradiated WBC isolated from whole blood and identified radiation-responsive genes up to 72 h after exposure (13). More recently, using *ex vivo* irradiated whole blood, we identified a set of 74 genes that can predict radiation dose across a broad dose range at 6 and 24 h after exposure without the need for matched pre-exposure controls (14). To extend the potential usefulness of this approach and enable screening in the field, translation of our radiation signatures to a fully automated “lab-on-a-chip” device is currently in progress using a quantitative nuclease protection assay run directly on lysed blood from a fingerstick (15). The current prototype completes the assay in about 9 h, and both point-of-care and high-throughput approaches are being developed.

Although *ex vivo* studies are flexible and provide a convenient platform for gene discovery, it is obviously also important to confirm the findings in human populations exposed *in vivo* to the extent that this is possible. Our initial *in vivo* gene expression study looked at a limited number of patients undergoing total-body irradiation (TBI) prior to bone marrow transplantation (16). This study confirmed the *in vivo* response of many of the genes identified as radiation responsive *ex vivo* and also identified additional genes that had not responded in the *ex vivo* studies. More recently, a whole genome study using TBI patients (17,18) reported an 18-gene signature that predicted samples as either control or irradiated with 90% accuracy.

In the present study, we have applied whole genome microarray analysis to blood samples from a heterogeneous population of TBI patients and identified genes that respond to radiation exposure *in vivo*, many of which were not responsive to radiation in the *ex vivo* studies. We have also compared the performance of classifiers built from these genes with the performance of a classifier based on the 74-gene set identified from our *ex vivo* radiation studies (14). The classifiers derived from both the *in vivo* and the *ex vivo* radiation studies performed well in predicting exposure status of the samples as either pretreatment control, 1.25 Gy or 3.75 Gy, achieving up to 98% accuracy. Additional controls from healthy individuals did not confound the prediction of dose. These findings strengthen our approach of using *ex vivo* studies to model *in vivo* responses for doses that are not available in patients undergoing whole-body irradiation.

METHODS AND MATERIALS

TBI Patients and RNA Purification

Patients undergoing TBI at Memorial Sloan Kettering Cancer Center (MSKCC) prior to hematopoietic stem cell transplant were recruited with informed consent under a prospective protocol approved by the Institutional Review Boards of MSKCC and Columbia University Medical Center. TBI was delivered using high-energy photons (15 MV) produced by a linear accelerator. Patients were treated at a distance (440 cm from the source) using parallel-opposed anteroposterior beams to a total dose of 1.25 Gy per fraction, 3 fractions/day, at a dose rate of approximately 10 cGy/min, depending on body habitus and transplant protocol. Partial shielding was used to limit lung dose to about 8 Gy, with compensatory electron boost of the shielded chest wall (19). A total of 18 adult patients (12 males and 6 females), diagnosed with diseases including acute myelogenous leukemia, acute lymphocytic leukemia, chronic myelogenous leukemia, mantle cell lymphoma, non-Hodgkin's lymphoma, multiple myeloma, T-cell acute lymphocytic leukemia and aplastic

anemia participated in this study (Supplementary Table 1). None had prior radiation treatment or genotoxic drugs within 2 weeks before the start of radiation treatment. All patients were in remission, with no blast cells detected in the peripheral blood by conventional microscopy techniques; thus the gene expression profiles obtained represent healthy cells of cancer patients and not responses in cancer cells.

Peripheral blood was collected in PAXgene RNA tubes (PreAnalytiX GmbH) before irradiation, at 4 h after the first 1.25-Gy fraction, and at 20–24 h after the first fraction. The 1-day samples included exposure to three 1.25-Gy fractions with approximately 4 h between fractions for a total dose of 3.75 Gy. RNA extraction, globin reduction and quality control were performed as described (14). Blood from 14 healthy controls (7 males and 7 females) was also processed identically. All RNA samples had RNA integrity numbers between 8 and 9.9 (mean 8.84) and 260/280 absorbance ratios between 1.95 and 2.17 (mean 2.04).

Quantitative RT-PCR

Gene-specific primers and probe sequences were as reported previously (14), with additional primer-probe sets *ACTB* (forward: 5'-CACTCTTCCAGCCTTCCTTC, reverse: 5'-GGATGTCCACGTCACACTTC, probe: 5'-TGCCACAGGACTCCATGCCC), *DDB2* (forward: 5'-CAGGACACGGAAGTGAGAGA, reverse: 5'-CAAATCACACCTCTGCTTG, probe: 5'-TCCAAGGCCTTGTCTGGCCC), and *PCNA* (forward: 5'-ATTGCGGATATGGGACACTT, reverse: 5'-GCTGGCATCTTAGAAGCAGTTC, probe: 5'-TCATCCTCGATCTTGGGAGCCA) designed with Applied Biosystems' Primer Express (Applied Biosystems, Foster City, CA) and GeneScript Corporation's TaqMan Primer Design and synthesized by Operon Biotech, Inc. (Huntsville, AL). Reverse transcription and real-time PCR reactions were conducted using standard methods as described (14). All sample reactions were run in duplicate with three repeats on different days, with relative induction calculated by the $\Delta\Delta C_T$ method normalizing to *ACTB*.

Microarray Processing and Data Extraction

Agilent's One-Color Quick Amp labeling kit (Santa Clara, CA) was used to prepare Cyanine-3 (Cy3)-labeled cRNA using 0.5 μ g input RNA according to the manufacturer's instructions, followed by purification of cRNA by RNeasy column (Qiagen, Valencia, CA). Yield and Cy3 incorporation were checked using the NanoDrop ND-1000 Spectrophotometer. After labeling, 1.65 μ g cRNA was fragmented and hybridized to Agilent whole genome microarrays (G4112A) at 65°C for 17 h with rotation followed by washing as recommended by Agilent and scanning with the Agilent Scanner (G2404B) using the recommended settings. Images were analyzed with Feature Extraction 9.1 (Agilent) using default parameters for background correction and flagging of nonuniform features.

Microarray Data Analysis Using BRB Array Tools

Background-corrected hybridization intensities were imported into BRB-ArrayTools Version 3.8.0 (20), log2-transformed and median normalized. Six-hour *ex vivo* gene expression profiles from prior experiments with the blood of healthy individuals (14) were imported for comparison. Nonuniform outliers or features not significantly above background intensity in 25% or more of the samples and features not changing at least 1.5-fold in at least 20% of the samples were filtered out, yielding 19,704 features that were used in subsequent analyses. The microarray data are available through the Gene Expression Omnibus using accession number GSE20162.

Genes that were differentially expressed in irradiated and unirradiated samples were identified with BRB-ArrayTools, using random variance *t* or *F* tests. Genes with *P* values less than 0.001 were considered statistically significant. The false discovery rate (FDR) was also estimated for each gene using the method of Benjamini and Hochberg (21) to control for false positives.

BRB-ArrayTools was also used to visualize the data by multidimensional scaling (MDS) and to build classifiers for predicting the radiation dose category of each sample using diagonal linear discriminant analysis, 3-nearest neighbor, and nearest centroid algorithms. Genes differentially expressed with a univariate parametric significance level $P < 10^{-7}$ were used, and the cross-validated prediction error was estimated using a leave-one-out approach.

Gene Ontology Analyses

The significantly differentially expressed genes were imported into PANTHER (22) and the number of genes in each functional classification category was compared against the number of genes in that category in the NCBI human genome. The binomial test was used to determine statistical over-representation of PANTHER classification categories (23). Bonferroni-corrected $P < 0.05$ were considered significant.

RESULTS

Microarray Experiments

Global gene expression in WBC of 18 patients undergoing TBI with 1.25-Gy radiation fractions was measured at 4 h or 1 day after the beginning of TBI. Later times were not sampled due to increasing difficulties of interpreting data after accumulation of multiple fractions. The first post-treatment samples received a single 1.25-Gy fraction, while the second post-treatment blood draws had an accumulated dose of 3.75 Gy (three 1.25-Gy fractions). The Agilent one-color workflow was used to hybridize whole genome microarrays to facilitate the identification of genes without relying on paired expression ratios with matched pre-exposure controls. This approach is important to enable translation to a radiation triage situation, because in such situations pre-exposure controls would not be available. The microarray data were filtered for quality and minimum change between dose classes prior to analysis.

Identification of Differentially Expressed Genes

We used the Class Comparison feature of BRBArrayTools to identify genes that were differentially expressed after one and three fractions of TBI. After the first 1.25-Gy fraction, 692 genes were differentially expressed ($P < 0.001$ and $\text{FDR} < 2.9\%$; Supplementary Table 2). After the accumulation of 3.75 Gy, 1588 genes were differentially expressed ($P < 0.001$ and $\text{FDR} < 1.3\%$; Supplementary Table 3). We next looked for genes with dose-dependent expression across all three doses (0 Gy, 1.25 Gy and 3.75 Gy), identifying 1523 differentially expressed genes ($P < 0.001$ and $\text{FDR} < 1.3\%$; Supplementary Table 4). Comparing these lists, 413 genes were significantly differentially expressed in all three analyses (Supplementary Table 5).

Gene Ontology Analysis of Genes Responding In Vivo

The three lists of differentially expressed genes, those responding after 1.25 Gy or 3.75 Gy or the dose-dependent genes, were analyzed for enrichment of gene groups using PANTHER (23). The PANTHER approach uses protein sequence information and gene families to assign a gene to an ontology group. The most significantly over-represented biological processes in all three analyses were immunity and defense, followed by signal transduction and apoptosis. Although these three processes were also over-represented

among genes responding to *ex vivo* irradiation, many more genes in these categories responded *in vivo*, resulting in much smaller enrichment *P* values (Table 1). Many of the other significant biological processes affected by TBI represented more specialized subsets of these categories, such as cytokine- and chemokine-mediated signaling pathway, T-cell-mediated immunity, NK cell-mediated immunity, and other signaling pathway-related categories.

Validation of Microarray Gene Expression

We used quantitative real-time RT-PCR (qRT-PCR) to validate the microarray results for seven genes (*CDKN1A*, *FDXR*, *BBC3*, *PHPT1*, *SESNI*, *DDB2* and *PCNA*) that responded by microarray both in TBI patients and in our previous *ex vivo* irradiation studies. The mean ratios of gene expression in TBI patients compared with their matched preirradiation controls were calculated for these seven genes using the qRT-PCR data and compared with the ratios calculated from the microarray data (Fig. 1), showing good overall agreement between the two measurement techniques.

Prediction of Radiation Dose to Individual Samples

Because an overall goal of our ongoing work is to develop signatures predictive of the level of radiation exposure, not simply to identify differentially expressed genes, we used the microarray expression data to build and test classifiers using leave-one-out cross-validation with three different algorithms [diagonal linear discriminant analysis (DLDA), 3-nearest neighbor (3-NN), and nearest centroid (NC)]. Classifiers built with the microarray data from TBI patients predicted the dose category of an individual sample as 0, 1.25 or 3.75 Gy correctly for 96% of samples by all three algorithms using a set of 152 genes. The permutation *P* value of the cross-validated misclassification error rate was <0.001.

The patients undergoing TBI had a range of different diagnoses and had been subjected to many different chemotherapy regimens and conditioning regimens prior to the start of irradiation. Since their control level of gene expression might be expected to vary considerably from that of healthy individuals, we also included additional healthy controls, consisting of unirradiated blood drawn from healthy donors and processed identically to the patients' blood. When we tested our classifiers on this larger set, the algorithms still performed well, providing 83–94% correct dose prediction of all samples, regardless of health status.

In our previous study using *ex vivo* irradiation of human peripheral blood as a model for *in vivo* irradiation we reported a signature of 74 genes that performed well at times from 6–24 h after exposure and across doses from 0 to 8 Gy (14). To see how well that *ex vivo* selected signature could predict dose in the TBI patients, we next built classifiers using the same algorithms and the 74-gene set and obtained 96–98% correct prediction of the exposure levels of the TBI patient samples. The 74-gene set showed no erosion of performance when the samples from the healthy donors were included, predicting the dose correctly in 98% of the samples by all methods. A comparison of the performance of the 3-NN classifiers built using the TBI data or the *ex vivo* signature is shown in Table 2 for class prediction of all samples (TBI patients and healthy donors).

DISCUSSION

Since the early application of microarrays to radiation responses in human WBC first suggested the potential of gene expression for biodosimetry (13), studies have continued to support this application. Gene expression signatures in peripheral WBC can predict radiation dose using both *ex vivo* and *in vivo* models (14,17,18). In the present study we tested the

ability of gene expression profiles to predict radiation doses received *in vivo* in a heterogeneous population of TBI patients and to compare the predictive ability of signatures derived from *in vivo* versus *ex vivo* exposures. Classifiers built from *in vivo* responding genes were able to predict radiation exposure levels of the *in vivo* samples with 96% accuracy. Classifiers built from the gene signature identified previously in *ex vivo* experiments (14) were similarly effective, predicting the dose to TBI samples with 98% accuracy, indicating that the *ex vivo* signature is robust across different sample sets and radiation models.

A potential concern in the use of cancer patients for these studies is the possibility that their clinical conditions or prior treatments could cause alterations in preirradiation gene expression that could confound radiation signatures. The heterogeneity of diagnoses in this study should buffer somewhat against selecting gene expression signatures that would apply only to a subset of individuals (those either predisposed to or having a specific disease). We added to our analysis 14 control samples from healthy donors. Adding these samples did not dramatically affect the predictive ability of either the TBI or *ex vivo* derived classifiers. Using the 3-NN algorithm, the signature derived from the TBI data predicted the dose category of 94% of the samples (including the healthy controls) correctly, and the *ex vivo* derived signature predicted 98% of the samples correctly (Table 2). Although we cannot directly test samples from healthy people irradiated *in vivo*, this study demonstrates that there is no appreciable confounding between unirradiated healthy people and cancer patients.

Comparing the current findings with our first analysis of *in vivo* radiation response (16), only a subset of the genes responding significantly in the current study were identified in that earlier study. The first study surveyed only 6485 genes, used different protocols, and included microarray data from only one patient. Studies of radiation responses in the skin of radiotherapy patients have found large variations in the specific genes responding in individual patients, but in that case gene set enrichment analysis indicated conserved functions among the responding genes (24,25).

Gene ontology analysis of our earlier *in vivo* study had identified immune and inflammatory responses as the most significant processes represented among the radiation-responsive genes, a finding consistent with the gene ontology analysis of the present study (Table 1). After updating the gene annotations using the Array Clone Information Database (26) we analyzed the older TBI gene set using PANTHER (23). Despite the smaller number of genes in the earlier study, we found similar enrichment of immune- and cytokine-related responses in the two studies, consistent with the profound immunomodulatory effects of TBI observed in hematopoietic stem cell transplantation (27).

These immune-related categories were also enriched in the analysis of data sets from our *ex vivo* study (14). In contrast to the TBI study, the *ex vivo* response was dominated by genes associated with apoptosis, cell cycle and the p53 pathway, rather than with immune functions, consistent with the responses in cell lines irradiated *in vitro* (28). Because our TBI study spanned only 0–3.75 Gy, while the *ex vivo* study was based on genes with dose-dependent responses up to 8 Gy, the use of higher doses may also contribute to relative differences in gene ontology enrichment. It is important to note that the p53-related functions were also represented among the significant TBI responses; they were just not the most highly enriched categories. The broad patterns of gene categories responding *in vivo* and *ex vivo* highlight the similarity between whole organism stress responses and cellular responses *in vitro*. They suggest a “core” of gene expression responses, many of which are conserved *ex vivo*, with additional responses and layers of signaling present in the whole organism. The greater magnitude of the *in vivo* response was reflected in the larger numbers

of genes responding significantly *in vivo* in all the categories examined. This supports our earlier suggestion that *ex vivo* selection of radiation signature genes may actually help reduce the potential for confounding by excluding a number of immune response genes that respond robustly to radiation *in vivo* but that may represent more generalized stress responses not specific for radiation exposure.

While it should be kept in mind that the response of a signature is likely to be less prone to confounding than the response of an individual gene from that signature (29), extensive testing for potential confounding of dosimetric signatures will ultimately be needed. Variation is known to exist in the peripheral blood gene expression profiles of healthy individuals (30,31), and subtle variations may be attributed to factors such as body mass index or smoking status (32). Acute or chronic disease states including infection (33), sepsis (34,35), immune or inflammatory conditions (36), stroke (37), cancer (38) and transplant rejection (39) can also alter the peripheral blood gene expression profile.

Injuries such as burns or physical trauma also produce specific gene expression signatures in peripheral blood, and these would be especially relevant in the context of a radiological or nuclear event. To get some preliminary gauge of the amount of overlap that might be expected in responses to such trauma and the response to ionizing radiation exposure, we compared our *ex vivo* radiation signature with the genes reported to respond significantly at 1 day after treatment in a recent study comparing gene expression changes in the blood of mice undergoing traumatic blood loss or scald burns over 25% of their bodies (40). Of 4850 genes reported as responding in the burn model at 1 day after injury, 9 genes (*BTG3*, *CDKN1A*, *GADD45A*, *LY9*, *MDM2*, *MGAT3*, *PLK2*, *PTP4A1* and *SESN1*) changed in the same direction in our radiation signature. A further 6 genes from our signature (*ARHGEF3*, *DDB2*, *IL21R*, *LIG1*, *PHPT1* and *SLC7A6*) were upregulated by radiation but downregulated in the burn study. In the trauma-hemorrhage study 1164 genes responded 1 day after blood loss. Of these, five genes from our *ex vivo* signature (*BTG3*, *CCNG1*, *CDKN1A*, *DCPIB* and *TM7SF3*) were upregulated both by blood loss and by radiation exposure, and one gene (*DDB2*) was downregulated by blood loss but upregulated by radiation. The results of this comparison illustrate some of the complexities of gene expression responses to different stresses and suggest that although some genes in our current radiation signature will respond similarly to traumatic injuries, the majority of the signature is not likely to overlap with the response to a different specific stressor, and most importantly, the overall patterns of response are likely to be distinct. Further direct testing, not only comparisons with published studies, will obviously be required to fully assess the extent to which conditions of trauma, disease or lifestyle factors may affect the actual estimation of radiation dose using gene expression.

We also compared our TBI results with the predictive signature reported by Dressman *et al.* (17). These authors identified 25 probe sequences representing 18 human genes that predicted radiation exposure between pre-TBI and post-TBI samples with 90% accuracy. Of these genes, 16 were significantly regulated in our current TBI study and 13 were included in our *ex vivo* signature. Although we had focused on prediction between three sample classes (unexposed, 1.25 Gy or 3.75 Gy), it was of interest to compare the ability of our approach to predict samples as either pre- or postexposure, either after the first fraction as reported (17) or for any unexposed versus any postexposed. Focusing on the first dose alone also avoids complications in interpretation that may arise due to the three-fraction nature of the 3.75-Gy total-body dose. Classifiers built either from our TBI data or using the previously defined *ex vivo* signature predicted the samples (including the healthy controls) as exposed or unexposed with 100% accuracy by all methods tested.

The separation of control and *in vivo* irradiated samples using the *ex vivo* signature is illustrated by multi-dimensional scaling (MDS) in Fig. 2. MDS is a visualization tool that preserves pairwise similarities between samples while allowing graphical representation of high-dimensional data in two or three dimensions (41). Each point represents a sample and the first three principal components of the gene expression matrix are used as axes. The distance between any two points represents the overall similarity of the expression levels of all genes in the signature. The similarity between controls (from healthy donors or TBI patients) further illustrates that the disease status of TBI patients does not detrimentally affect the baseline expression of the genes in our *ex vivo* signature. The 6-h control and 2-Gy samples or 24-h control and 5-Gy samples from our previous *ex vivo* study (14) are included in the MDS for comparison. A similar separation is seen between control and irradiated samples both *in vivo* and *ex vivo*, although a lateral shift is also evident between *in vivo* and *ex vivo* samples in the MDS (Fig. 2). This lateral shift suggests that *ex vivo* incubation did affect expression of some genes in the signature independent of radiation exposure, as previously reported for increasing time in culture (14). The “time in culture” effect did not interfere with the prediction of radiation dose in either study, however. Previous studies have also demonstrated some gene expression changes in WBC during *ex vivo* incubation (42).

Because our previous signatures have all incorporated information from more than the 25 probe sets used in ref. (17), we were also interested to see if we could achieve good classification of samples using smaller gene sets. To this end, we applied a support vector machine recursive feature elimination algorithm in BRB-Array-Tools (20) to select 25 features for predicting the status of the unexposed or post-first-fraction samples, analogous to the Dressman study. This smaller feature set (Supplementary Table 6) also yielded 100% correct prediction of exposure status.

Inter- and intraindividual variations in gene expression do occur among healthy individuals (31,43), and some genes respond to radiation with great variability in immortalized blood cells derived from different donors (44), suggesting a potential problem for accurate prediction of radiation exposure by gene expression profiling. In practice, however, we find that appropriate signature selection can provide accurate prediction of both exposure status and dose. Using a signature of multiple genes also “buffers” the predictive ability by reducing the contribution of individual variations that may occur in a single gene. This has been reported with other biodosimetry approaches, where combining measurement of up to three serum proteins (45) or adding C-reactive protein measurements to time-dependent blood cell counts (29) improved dose prediction or identification of irradiated samples and provided robustness against confounding. Nonetheless, more extensive testing on large heterogeneous populations will still be needed prior to final implementation of any gene expression signature for radiation biodosimetry.

The performance of our *ex vivo* signature in predicting dose classes of samples from TBI patients irradiated *in vivo* further supports the robust nature of this signature, because it performs well not only under different irradiation conditions but also when applied to a completely different population with different health status from that in which it was selected. This signature predicted exposure with 100% accuracy and dose level with up to 98% accuracy in a heterogeneous human population undergoing TBI with variable gender, age and prior treatment history without the need for a pre-exposure control. These findings strongly support the utility of further developing this approach to provide biodosimetry in the event of a deliberate or accidental radiological or nuclear event.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIG. 1.

Expression of individual genes relative to pre-TBI controls (panel A) after one 1.25-Gy fraction of TBI or (panel B) after 3.75 Gy (3 fractions). White bars represent the means and standard error of qRT-PCR measurements for all patients; black bars are microarray measurements.

FIG. 2.

MDS plots of pre- and post-TBI samples using the *ex vivo* gene signature. Each point represents an individual sample; the axes represent the first three principal components of the MDS. Dark blue: patients prior to TBI; cyan: healthy unirradiated donors processed as *in vivo* samples. Panel A: The early responses to radiation in the blood of patients 4 h after the first (1.25-Gy) TBI fraction (purple) and in samples from healthy donors that were incubated 6 h *ex vivo* after no irradiation (green) or 2 Gy γ rays (red). Panel B: Responses 24 h after radiation exposure in the blood of patients exposed to three TBI fractions (pink; total dose of 3.75 Gy) and in samples from healthy donors that were incubated 24 h *ex vivo* after no irradiation (light green) or 5 Gy γ rays (orange).

TABLE 1
PANTHER Gene Ontology Analysis of Significantly Over-represented Biological Processes after *In Vivo* and *Ex Vivo* Irradiation

Biological process (no. genes in reference set)	<i>In vivo</i> TBI <i>P</i> values			<i>Ex vivo</i> <i>P</i> values		
	3 Dose ^b	3.75 Gy	1.25 Gy	24 h	6 h	
Immunity and defense (1318)	1.88 × 10 ⁻²⁰ 136 (55)	5.25 × 10 ⁻¹⁷ 131 (57)	1.05 × 10 ⁻¹⁴ 73 (25)	0.0275 14 (5)	NS 24 (13)	
Signal transduction (3406)	1.71 × 10 ⁻¹² 230 (141)	2.38 × 10 ⁻¹⁰ 228 (147)	0.000265 99 (64)	0.0121 27 (14)	0.000455 59 (34)	
Apoptosis (531)	7.84 × 10 ⁻⁷ 52 (22)	2.46 × 10 ⁻⁸ 57 (23)	1.45 × 10 ⁻⁷ 33 (10)	0.00197 10 (2)	0.0000172 20 (5)	
Nucleoside, nucleotide and nucleic acid metabolism (3343)	8.92 × 10 ⁻⁷ 202 (139)	4.91 × 10 ⁻⁹ 219 (144)	0.0172 89 (63)	NS 21 (14)	NS 47 (33)	
Intracellular signaling cascade (871)	1.61 × 10 ⁻⁶ 74 (36)	1.48 × 10 ⁻⁷ 79 (37)	0.000859 37 (16)	NS 9 (4)	NS 20 (9)	
T-cell-mediated immunity (194)	3.98 × 10 ⁻⁶ 28 (8)	1.02 × 10 ⁻⁸ 33 (8)	NS 12 (4)	NS 2 (1)	NS 7 (2)	
Cytokine and chemokine-mediated signaling pathway (252)	0.000102 30 (10)	0.000213 30 (11)	0.000259 20 (5)	NS 3 (1)	NS 8 (3)	
Natural killer cell-mediated immunity (74)	0.000121 15 (3)	0.000188 15 (3)	NS 3 (1)	NS 2 (0)	NS 1 (1)	
Protein modification (1157)	0.000409 82 (48)	0.000498 84 (50)	NS 36 (22)	NS 9 (5)	NS 23 (12)	
Ligand-mediated signaling (421)	0.000414 40 (17)	0.005 38 (18)	NS 17 (8)	NS 5 (2)	NS 4 (4)	
Cell cycle control (418)	0.000614 39 (17)	0.000424 43 (18)	0.00974 21 (8)	NS 6 (2)	NS 12 (4)	
Cell surface receptor-mediated signal transduction (1638)	0.000802 106 (68)	NS 99 (70)	0.0167 53 (31)	NS 12 (7)	0.00681 34 (16)	
Developmental processes (2152)	0.000942 128 (89)	0.000166 136 (93)	NS 55 (41)	NS 13 (9)	NS 30 (21)	
Cell cycle (1009)	0.0016 69 (42)	2.09 × 10 ⁻⁷ 85 (43)	NS 30 (19)	NS 10 (4)	0.00727 23 (10)	
Cell proliferation and differentiation (1028)	0.00161 70 (43)	0.000169 76 (44)	0.0223 35 (19)	0.00223 14 (4)	0.0229 22 (10)	
B-cell- and antibody-mediated immunity (97)	0.00293 15 (4)	0.0164 14 (4)	0.0175 9 (2)	0.00751 5 (0)	NS 6 (1)	
Cell communication (1213)	0.00379 81 (50)	NS 76 (52)	NS 34 (23)	NS 8 (5)	NS 18 (12)	
Oncogenesis (472)	0.00381 38 (20)	NS 35 (20)	0.0015 23 (9)	NS 5 (2)	NS 11 (5)	
Protein phosphorylation (660)	0.00526 51 (27)	0.00177 54 (28)	NS 22 (12)	NS 8 (3)	0.0275 18 (7)	
Chromatin packaging and remodeling (237)	0.0124 24 (10)	0.00115 27 (10)	NS 4 (4)	NS 0 (1)	NS 1 (2)	

^aData for analysis of *ex vivo* biological processes from ref. (14).

^bNumbers on the first line are *P* values of significance of category enrichment, NS: not significant (*P* > 0.05). Numbers on the second line are the number of significantly responding genes belonging to each category followed by the number expected in that category by chance (in parentheses) given the number of genes responding to a specific condition. See refs. (22, 23) for details of gene ontology analyses and statistics.

TABLE 2A

Performance of 3-NN Classifiers on all Samples (TBI plus healthy controls)

Dose (Gy)	Sensitivity	Specificity
<i>In vivo</i> signature		
0	0.969	0.941
1.25	0.833	0.958
3.75	0.875	0.92
74-gene <i>ex vivo</i> signature		
0	1	1
1.25	1	0.979
3.75	0.938	1

TABLE 2B

Performance of 3-NN Classifiers on Postirradiation Samples Only

Dose (Gy)	Sensitivity	Specificity
<i>In vivo</i> signature		
1.25	1	0.875
3.75	0.875	1
74-gene <i>ex vivo</i> signature		
1.25	0.944	0.938
3.75	0.938	0.944