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TRANSIENT GENOME-WIDE TRANSCRIPTIONAL RESPONSE TO LOW-DOSE IONIZING RADIATION *IN VIVO* IN HUMANS

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Abstract

Purpose—The *in vivo* effects of low-dose low linear energy transfer ionizing radiation on healthy human skin are largely unknown. Using a patient-based tissue acquisition protocol, we have performed a series of genomic analyses on the temporal dynamics over a 24-hour period to determine the radiation response after a single exposure of 10 cGy.

Methods and Materials—RNA from each patient tissue sample was hybridized to an Affymetrix Human Genome U133 Plus 2.0 array. Data analysis was performed on selected gene groups and pathways.

Results—Nineteen gene groups and seven gene pathways that had been shown to be radiation responsive were analyzed. Of these, nine gene groups showed significant transient transcriptional changes in the human tissue samples, which returned to baseline by 24 hours postexposure.

Conclusions—Low doses of ionizing radiation on full-thickness human skin produce a definable temporal response out to 24 hours postexposure. Genes involved in DNA and tissue remodeling, cell cycle transition, and inflammation show statistically significant changes in expression, despite variability between patients. These data serve as a reference for the temporal dynamics of ionizing radiation response following low-dose exposure in healthy full-thickness human skin.

Keywords

Low-dose ionizing radiation; Biosignature; Human; Genomic expression patterns; *In vivo* dosimetry

INTRODUCTION

The effects of low-dose low linear energy transfer ionizing radiation (LDIR) in humans are of growing concern, especially in the context of current radiation techniques such as intensity-modulated radiation therapy (IMRT) and medical imaging. The biological response of healthy tissue to low doses of 1–10 cGy *in vivo* is unknown. Because of ethical considerations *in vivo* studies have been hindered because it is not possible to irradiate otherwise healthy individuals solely to study the human response to LDIR. There are no data examining the acute,

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transcriptional changes in normal tissue response at the lower doses received outside of the primary treatment field. These surrounding tissues are at risk for late normal tissue complications. The amount of tissue receiving low-dose exposures is increasing given the use of IMRT, for which a more conformational treatment of the target tissue results in additional scatter dose associated with substantially longer beam times.

Therapeutic radiation is used to treat a variety of malignancies, either as the primary treatment or in an adjuvant setting. The treatment strategies have evolved over time on the basis of clinical trials and evaluation of maximally tolerated doses of radiation on normal tissues. A standard time between doses (or fractions) of radiation therapy is 24 hours, based on workday scheduling and empiric observations. There has been an assumption that the immediate effects of each fraction would have returned to baseline before the next treatment, but this has not been evaluated outside of the repair kinetics for the spinal cord at the moderate ionizing radiation (IR) dose of approximately 2 Gy (1–3). Although the intratumoral treatment dose is usually 1.8–2.0 Gy/fraction, the surrounding normal tissues outside of the tumor receive lower doses, with some tissues receiving very low-dose exposures (4). Previous work from this group (4, 5) has detailed a methodology for using the lower dose exposure areas on the skin surface as an accessible human tissue model for biologic sampling for low-dose radiation biology studies (4,5).

Response to LDIR has been studied in animal models, keratinocytes, fibroblasts, lymphocytes, and other cell lines, and differing, independent profiles are seen in the cellular response to exposure to either low or high doses of ionizing radiation (6–11). Yin *et al*. (12) examined brain tissue derived from low- and high-dose full-body IR exposures and demonstrated that each response was qualitatively different from the other. These studies found that the transcriptional profiles could be categorized into three main groups: those that were altered by low- and high-dose exposures, those that were unique to low dose, and those unique to high-dose exposures. These data highlight the fact that low-dose and high-dose radiation responses are biologically diverse. In the era of IMRT, it is possible that late normal tissue responses from low-dose exposure only may be of a profile different from those arising in the high-dose region. These differing profiles may reflect entirely different processes, not simply a lesser degree of the same response.

Identifying the low-dose temporal response profile is of medical importance and has implications for counseling patients undergoing radiation therapy. Interest in normal tissue complications have increased because of the growing number of patients who have undergone ionizing radiation therapy and now have extended life expectancies (13). Furthermore, attempts to alter the time between fractions to increase the efficacy of treating the cancer have shown promise, but there are no data on how this might affect healthy tissue tolerance, especially for the tissue outside the targeted treatment field (14). Additionally, radiation response profiles are also now of national strategic interest with the growing concern for potential population exposure to low levels of radiation via terrorist acts involving radiation dispersal devices (as reviewed in 15–17). Thus, for both individual cancer patient counseling and public policy development, information on the temporal response to low-dose radiation exposure in human tissue is needed.

This study begins to address the information gap of human response to LDIR. We have developed a model for direct evaluation of the effects of LDIR in normal, healthy human tissue. Initial studies using this approach in conjunction with a statistically valid data analysis model have been used to evaluate the dose response profile at 3 hours postexposure (18–20). The studies presented herein describe the human responses to equal doses of LDIR over a 24-hour period following a single radiation exposure. By analyzing the response pattern over this

period, we have been able to develop quantitative data on normal human skin responses that can be used as a benchmark for evaluating LDIR temporal patterns.

METHODS AND MATERIALS

Dosimetry

During patient treatment planning, an extra CT scan was obtained in treatment position to be used for biopsy planning. PEREGRINE Monte Carlo dose calculations were used to determine the biopsy sites to receive 10 cGy. There is variability with the 10 cGy dose because this point is located at the edge of the treatment area where there is a steep dose gradient around the biopsy location. Patient breathing as well as minimal movement can result in dose changes (4). A procedure was created using a linear array of metal oxide semiconductor field effect transistor (MOSFET) detectors for each biopsy location. The MOSFET array (5 MOSFETs, 1-cm intervals) was placed on the anterior abdominal wall, positioned to cover the expected 10 cGy location (at the edge of the exit beam of the posterior treatment field). Tissue-equivalent bolus material was placed on the abdominal skin where biopsies were to be taken to ensure that the tissue was in electronic equilibrium, thereby reducing the absorbed dose variability in the sample. MOSFETs were read after treatment while the patient was still in position on the linac couch. Interpolation between the five MOSFETs of each linear array was performed to identify the 10 cGy location. The MOSFET measurement is real-time dosimetry and incorporates all patient motion during the treatment, thus reflecting actual dose delivered. On the basis of our physics validation studies for MOSFETs, the uncertainty was below 15% for 1 cGy and below 10% for 10 cGy.

Biopsy procurement

Biopsy samples were obtained from a cohort of men with prostate cancer at the beginning of their radiation therapy. The men constitute a distinct group of patients from those in our dose-response study. Informed consent was obtained (Institutional Review Board approved following the Helsinki principles with Health Insurance Portability and Accountability Act compliancy). Biopsy points were identified using the MOSFETs and marked on the skin at the first treatment day. Each patient was treated with X-rays generated on a Varian 2100C (energy, 18 MV; dose rate, 600 cGy/min). A five-field isocentric treatment was used (isocenter at 100 cm). The treatment duration, with gantry movements between the fields, was approximately 5–10 min. Biopsies were performed on healthy abdominal skin at the prespecified time, and each sample was placed in 1 mL of RNAlater (Ambion, Austin, TX) immediately after extraction. Each man underwent four full-skin-thickness punch biopsies, including the underlying adipose layer, 3 mm in diameter. The mass of each biopsy was 19 mg on average. For each patient, the first biopsy was taken before any treatment to serve as the control. The remaining three samples were obtained at 3, 8, and 24 hours after the radiation exposure. Biopsies were stored at −20°C until further processing. All men in the study responded clinically to treatment. Long-term clinical follow-up has not been completed.

RNA extraction and labeling

All biopsy samples were processed simultaneously according to a modified trizol protocol (5). Briefly, each biopsy sample was lysed in 1 mL of a guanidine thiocyanate solution using a Fastprep 120 beadbeater (MP Biochemicals, Solon, OH). RNA was precipitated, resuspended, and cleaned using RNeasy columns (Qiagen, Valencia, CA). Residual DNA contamination was removed using Turbo DNA-Free (Ambion, Austin, TX). All RNA samples were stored at −80°C.

RNA was processed for Affymetrix arrays as previously described (18). In summary, 500 ng of mRNA was used in the first strand synthesis using the One-Cycle *in vitro* Transcription

Labeling kit (Affymetrix, Santa Clara, CA). Second strand synthesis was performed and resulting biotinylated cRNA targets were fragmented using standard Affymetrix protocols. For each sample, 500 μg of cRNA was hybridized to a Human Genome U133 Plus 2.0 array (Affymetrix, Santa Clara, CA).

Statistical analysis

Full details are given in reports by Rocke and colleagues (19,20). To summarize, for each probe set and individual patient, a *t* test was conducted to determine the difference between the 3- and 8-hour measurements versus the 0- and 24-hour measurements. This analysis tested for the presence of transient up-regulation, transient down-regulation, or neither, an approach that was taken to find transient responses that return to baseline by 24 hours. The *t* score for each probe set and patient will be positive if there is a trend toward transient up-regulation and negative if there is a trend toward down-regulation. To investigate the possibility of a weak signal in either direction, the entire data set of *t* scores was examined for a gene group or pathway. The hypothesis that the collection of *t* scores had a median of zero was tested using the Wilcoxon rank–sum test. If this was rejected, it indicated a trend toward transient up-regulation if the *t* scores were biased in a positive direction or toward down-regulation if the *t* scores were biased in a negative direction. The empirical *p* value (always two-sided) is the fraction of cases in the resampled gene sets in which the Wilcoxon test statistic was more significant than the actual test statistic from the gene group or pathway.

Gene group and pathway selections

Data analysis was based on biopsies taken from five patients at five time points, for a total of 20 arrays including five control samples. The samples were taken from healthy abdominal skin that received a 10 cGy dose verified by MOSFET readouts. To deal with the complexity of the data generated, we selected specific gene groups and pathways to be examined on the basis of the published literature in radiation response. The resulting 19 gene groups included the following: BCL2 associated athanogenes (BAG), BCL B-cell CLL/Lymphoma 2 (BCL2), BCL B-cell CLL/Lymphoma 6 (BCL6), bone morphogenic proteins (BMP), BMP receptor, cyclins, cytokines, growth arrest and DNA damage inducible protein 45 (GADD45), heat shock proteins (HSP), interleukin, keratins, mitogen-activated protein kinase (MAPK), protein disulfide isomerase (PDI), RAD51 homolog (RecA homolog, *E.coli*) (*S. cerevisiae*) (RAD51), S100, serine/threonine kinase, tumor necrosis factor (TNF), topoisomerase, and zinc finger proteins. Each of these gene groups was a compilation of many probes on the array, from 6 in the GADD 45 group to 799 in the zinc finger protein group. All of the probes encompassing each gene group can be found on the authors' web site (http://dmrocke.ucdavis.edu), and a detailed discussion can be found in Goldberg *et al*. (18).

On the basis of the *in vitro* and animal model literature, gene pathways were chosen that were considered to be radiation responsive. Because the rate of transcriptional response varies across the population as well as among our individual patients, we analyzed each patient for the entire gene pathway associated with the gene(s) of interest. Seven gene pathways were analyzed to capture the data from the sample cohort as a whole. The gene pathways included the following: Akt/PI3 kinase pathway (21–24), chemokine pathway (25,26), fibronectin pathway (23,25, 27), growth factor/insulin pathway (26), inflammation pathway (21,23,25,26,28,29), stress/ apoptosis pathway (21–23,25,26,28,30,31), and the transforming growth factor (TGF)-β/ cyclin/ubiquitin pathway (21,23–25,27,31–33). A detailed description of the rationale for the gene pathways selected and the pathways themselves is presented in the first publication of this model system. That study examined a dose-response question in a separate cohort of men and is available on the Web site of the second author (http://dmrocke.ucdavis.edu) (18).

RESULTS

In this component of the study, biopsies were collected before irradiation and at 3, 8, and 24 hours post-IR. Our analysis compared the transcriptional response at 3 and 8 hours with 0 and 24 hours. To detect transient responses that return to baseline by 24 hours post-LDIR exposure, we assumed that the 0-hour (pretreatment) control and the 24-hour time points would be significantly different from the tissue response at 3 or 8 hours postexposure. If transcripts were up-regulated or down-regulated at 3 and 8 hours compared with 0 and 24 hours, we detected a transient response that returns to baseline by 24 hours. The method of Rocke *et al.* (20) was chosen because of the small number of data points per patient, as well as previously observed variability in radiation response between individuals. This method is designed to detect differentially expressed gene groups and pathways based on the responses of multiple probe sets corresponding to the gene group or pathway. It allows us to check consistency by testing whether there is differential expression for each individual patient or collectively. The probe sets were summarized using the GLA expression index of Zhou and Rocke (34).

Gene groups and pathways were judged to be statistically significant if the two-sided resampling based empirical p value described in Methods and Materials was less than $p \leq 0.05$ (Table 1). We sampled 1,000 randomly chosen gene groups of the same size as the given gene group and ran the entire procedure on each grouping. The empirical p value was less than 0.05 if the number of test statistics from the random gene groups that are more extreme than the one calculated from the real gene group is less than 50 of the 1,000. The cited p values are the fraction of the random gene groups that gave more extreme statistics than the actual gene group. For example, for zinc finger proteins, none of the random groups generated a value as extreme as the actual zinc finger probe sets; for keratins, there were 40 of the 1,000 gene groups that generated a more extreme statistic. When none of the resample-based statistics exceeded the actual statistic, the p value was given as $p < 0.001$, which is the most significant result possible with this method. Details are given in Rocke *et al.* (20). It should be noted that another way to evaluate significance empirically is to permute the arrays. This may be more robust to correlations in the data than the method of resampling gene groups, but because of the small sample size, this is unusable in this data set. Figure 1 uses the zinc finger protein group data to illustrate the type of changes detected in this study.

Significant transient up-regulation was shown in zinc finger proteins ($p \sim 0$), keratins ($p = 0.040$), BMP receptor ($p = 0.028$), BAG ($p = 0.006$), and cyclins ($p = 0.016$). Significant transient down-regulation was detected in TNF ($p = 0.018$), interleukins ($p = 0.042$), heat shock proteins ($p \sim 0$), and S100 ($p = 0.040$) (Table 1). Ten of the gene groups did not show significant up- or down-regulation. The number of significant gene groups far exceeds the number expected by chance (9 were significantly differentially expressed vs. an expected number of <1). None of the pathways examined showed significant transient up-regulation or down-regulation as a whole. Tests of gene group responses in individual patients tended to show the same pattern of differential expression as seen across the whole patient cohort, when both are statistically significant (see Table 1 for details).

The microarray data from this study are available from the second author (http://dmrocke.ucdavis.edu) including the 20 .CEL files and the probe set summary data for all arrays and the 54675 probe sets in a Microsoft Excel file, as well as the experimental metadata. The data sets defining the gene groups and pathways, and the programs in the R language that were used to process the data are available on the same web site.

DISCUSSION

Low doses of IR have unknown biological consequences. In clinical radiation therapy, high doses of radiation are delivered to the target (tumor) tissue, whereas adjacent tissues receive lower doses. As more radiation therapy is delivered using highly conformal beam arrangements, such as IMRT, there are increases in the scattered dose of radiation to surrounding healthy tissue. With the surge in the number of cancer survivors, there comes a population of people who are living with the long-term effects of such radiation exposure. They are both a population of concern and one in whom biological studies can be undertaken to help broaden our understanding of the effects of low-dose radiation.

We have developed a model system that allows direct evaluation of radiation effects on healthy tissue by using techniques to confirm accurate dosimetry on individuals who are receiving localized therapeutic radiation for early-stage prostate cancer (4,18). This model allows for real-time sampling of human tissue after *in vivo* radiation exposures. This model is therefore unique in allowing the evaluation of whole tissue effects when exposure is under normal physiologic conditions. The full-thickness biopsy samples examined in these studies were evaluated as a whole tissue containing both epithelial and stromal cells. Therefore, the transcriptional profile is a comprehensive assessment of the responses of thousands of cells of multiple lineages, some classically “radiation sensitive,” whereas others would be “radiation resistant. ” Although specific cell-line outcomes cannot be identified, the tissue-level response is evaluated. Because the biomedical community is ultimately interested in defining risk to human tissues, the whole-tissue evaluation is a necessary level of study to begin to define human response to LDIR.

To study tissue-specific genomic responses to LDIR, gene groups and pathways known to be radiation sensitive were selected. These groupings are discussed in detail in Goldberg *et al*. (18), but in brief, they are involved in DNA repair, damage and remodeling (topoisomerase, zinc finger proteins), nuclear signaling, cell-cycle and associated check-points (cyclins, chk-1, chk-2, GRAP2, GPR51), inflammatory mediators (prostaglandin E2, cyclooxygenase-2, and interleukins), growth factors (epidermal growth factor receptor, tumor necrosis factor, vascular endothelial growth factor), apoptosis/survival signaling (Akt/phosphoinositide-3-kinase), and tissue structure and reorganization (keratins and ANLN).

Of the nine statistically significant groups, the zinc finger family of genes, the keratin gene group, and the cyclins were transiently up-regulated. Keratins are clearly tissue specific and suggest that there is repair or remodeling that is completed at the transcript level within 24 hours. Similarly, the cyclins and zinc finger proteins suggest that alterations in cell cycling and DNA remodeling are acute, transient responses to IR. Given that the doses examined are far below those that are frankly cytotoxic, this pattern of responses suggests that the tissue is actively undergoing some repair, even to such a low dose. The transient decrease in transcription of heat shock protein, TNF, and interleukin genes raises the possibility that the tissue attempts to diminish the acute stress response while it up-regulates transcription of DNA and tissue remodeling genes. Although this has not yet been evaluated in other human tissues, our results suggest that at least the skin response to LDIR may be substantially different from those seen following higher dose exposure. A response pattern of this type would be consistent with the emerging data from *in vitro* studies. The clinical implications of such a differing pattern have not yet been determined and will likely not be fully appreciated for many years to come.

The design of the study allowed for detection of *transient* up-regulation or down-regulation but not for detection of responses that are sustained beyond 24 hours. Given the positive gene group findings, as well as our previously reported positive low-dose radiation human skin biosignature data, the sustained nature of the tissue response seems a likely explanation for the

observation that none of the pathways were differentially expressed (18). The temporal dynamics of the transcriptional pathways we examined likely last longer than the 24-hour period of this data set. We are currently engaged in additional studies that will allow for detection of more sustained responses.

These data represent the first whole tissue, human temporal response data examining the effects of a single exposure to LDIR using precise dosimetry and statistically principled analyses. We have shown that it is possible to detect transient response to LDIR *in vivo* in humans and have identified nine gene groups that are either significantly up-regulated or significantly down-regulated. These data represent a reference library for genomic analysis of the temporal response of human skin exposed to a single dose of LDIR.

CONCLUSION

Low doses of ionizing radiation produce a definable temporal response within the first 24 hours after a single radiation exposure in full-thickness human skin. Genes involved in DNA and tissue remodeling, cell-cycle transition, and inflammation show statistically significant changes in expression, despite interindividual variability. These data have implications for therapeutic radiation schedules in which the interfraction interval is altered. This data set constitutes a reference group for temporal genomic analyses of LDIR in healthy, normal human skin.

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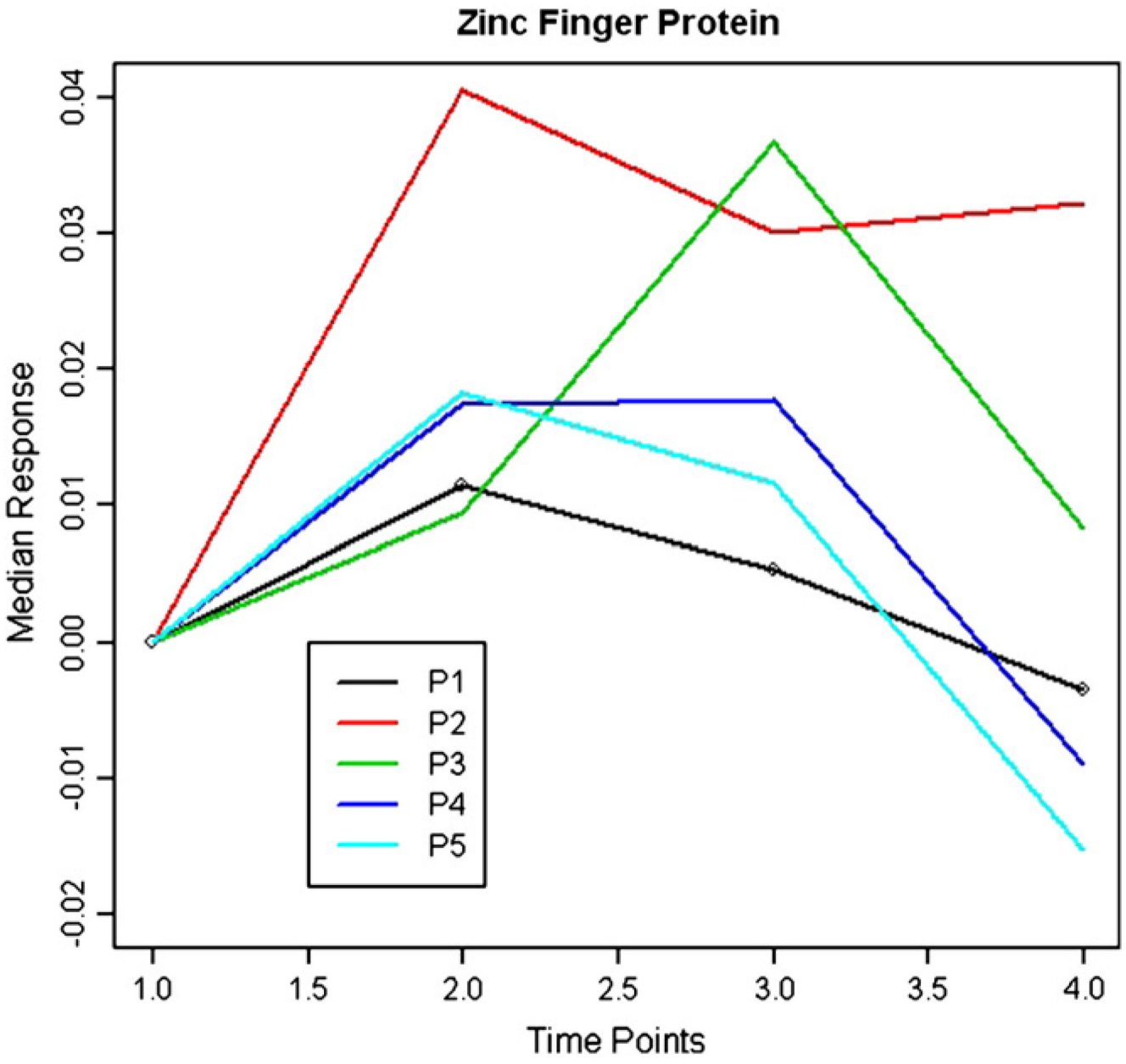

Fig. 1.

Time course of median transcriptional responses for the zinc finger protein gene group. Each line shows the change in expression from the initial time point, which is set to zero. The other time point values are differences in median expression between that time point and time 0. It can be seen that all patients exhibited a similar pattern of up-regulation followed by a falling off toward the original expression level. For this gene group, all five patients (P1–P5) showed transient up-regulation by our test, as did the cohort as a whole.

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Table 1

Results of statistical analysis of gene groups and pathways

Group or pathway	Probe sets	Overall (all patients)	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Groups							
BAG	**9**	**0.006 up**	0.376	0.000 up	0.046 up	0.500	0.556
BCL 2	47	0.996	0.758	0.054	0.904	0.796	0.396
BCL 6	8	0.084	0.144	0.952	0.238	0.760	0.038 up
BMPs	7	1.000	0.796	0.226	0.522	0.848	0.416
BMP Receptor	**8**	**0.028 up**	0.090	0.174	0.032 up	0.324	0.020 up
Cyclins	**123**	**0.016 up**	0.702	0.000 up	0.856	0.364	0.160
Cytokines	86	0.326	0.164	0.426	0.302	0.262	0.478
GADD45	6	0.162	0.936	0.288	0.078	0.502	0.182
HSP	**58**	**0.000 down**	0.422	0.960	0.000 down	0.000 down	0.004 down
Interleukins	**147**	**0.042 down**	0.788	0.222	0.352	0.006 down	0.224
Keratin	**101**	**0.040 up**	0.464	0.600	0.004 up	0.254	0.018 up
MAPK	131	0.974	0.078	0.890	0.064	0.376	0.548
PDI	8	0.060	0.262	0.008 up	0.000 down	0.152	0.006 down
RAD 51	10	0.564	0.584	0.520	0.730	0.880	0.520
S100	**21**	**0.040 down**	0.510	0.728	0.004 down	0.002 down	0.900
Serine/threonine kinase	73	0.704	0.488	0.302	0.964	0.518	0.388
TNF	**109**	**0.018 down**	0.484	0.946	0.572	0.004 down	0.178
Topoisomerase	14	0.614	0.548	0.266	0.878	0.980	0.398
Zinc finger	**799**	**0.000**	0.008	0.000	0.000	0.000	0.000

Group or pathway	Probe sets	Overall (all patients)	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
		up	up	up	up	up	up
Pathways							
Akt PI3 Kinase	99	0.114	0.104	0.394	0.530	0.844	0.336
Chemokines	79	0.530	0.608	0.880	0.306	0.602	0.140
Fibronectin	196	0.882	0.448	0.240	0.320	0.030	0.208
Growth factor/insulin	208	0.588	0.566	0.166	0.644	0.868	0.196
Inflammation	78	0.236	0.240	0.182	0.222	0.076	0.006
Stress/apoptosis	151	0.136	0.594	0.290	0.786	0.052	0.030
TGF-β/Cyclin/Ubiquitin	355	0.734	0.092	0.876	0.110	0.898	0.436

We tested for transient up-regulation or down-regulation by comparing the 3-hour and 8-hour expression values with the 0-hour and 24-hour expression values. If the 3-hour and 8-hour values were significantly higher than the 0-hour and 24-hour readings, we held that transient up-regulation or down-regulation had occurred. Up-regulation or down-regulation that persisted past 24 hours could not be detected by this test. Given the small number of data points for each gene (probe set), we analyzed the data for effects across groups of genes and pathways. Details are in Methods and Materials and the cited literature. Data are presented for individual patients, as are the Wilcoxon results for the entire temporal cohort, to investigate the zconsistency of the results across individuals and the whole group. Lines in bold face show gene groups and pathways that are significantly up-regulated or down-regulated for the entire cohort. The *p* values are empirical and based on resampling gene groups to show group trends as described in the text. Full gene names are listed in Methods and Materials