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TOPIC HIGHLIGHT

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Gene expression arrays as a tool to unravel mechanisms of normal tissue radiation injury and prediction of response

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Abstract

Over the past 5 years there has been a rapid increase in the use of microarray technology in the field of cancer research. The majority of studies use microarray analysis of tumor biopsies for profiling of molecular characteristics in an attempt to produce robust classifiers for prognosis. There are now several published gene sets that have been shown to predict for aggressive forms of breast cancer, where patients are most likely to benefit from adjuvant chemotherapy and tumors most likely to develop distant metastases, or be resistant to treatment. The number of publications relating to the use of microarrays for analysis of normal tissue damage, after cancer treatment or genotoxic exposure, is much more limited. A PubMed literature search was conducted using the following keywords and combination of terms: radiation, normal tissue, microarray, gene expression profiling, prediction. With respect to normal tissue radiation injury, microarrays have been used in three ways: (1) to generate gene signatures to identify sensitive and resistant populations (prognosis); (2) to identify sets of biomarker genes for estimating radiation exposure, either accidental or as a result of terrorist attack (diagnosis); (3) to identify genes and pathways involved in tissue response to injury (mechanistic). In this article we will review all (relevant) papers that covered our literature search criteria on microarray technology as it has been applied to normal tissue radiation biology and discuss how successful this has been in defining predisposition markers for radiation sensitivity or how it has helped us to unravel molecular mechanisms leading to acute and late tissue toxicity. We also discuss some of the problems and limitations in application and interpretation of such data.

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Key words: Radiation; Normal tissue response; Microarrays; Gene expression; Limitations

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MICROARRAYS FOR PREDICTION OF RADIOSENSITIVITY AND NORMAL TISSUE TOXICITY

The maximum dose of radiotherapy that can be given during cancer therapy is determined by the tolerance of normal tissues within the irradiation field. Patients vary considerably in their responses to radiation and it is the tolerance of the more sensitive subjects that limits the dose that can be given to the population as a whole; this may limit the chance of tumor cure in some cases. At least some of this variation in tissue response is genetically determined, which has stimulated research into the identification of those patients who are most susceptible to radiation damage, working towards individualization of dose prescription according to patient sensitivity[1-3]. The use of microarray technology to generate gene signatures for this purpose is a natural extension of earlier studies comparing radiosensitivity of cells (*e.g.* fibroblasts) from patients exhibiting severe and mild tissue damage. Although some of these studies did establish a correlation between *in vitro* radiosensitivity (clonogenic cell kill) and late normal tissue damage[4,5], larger studies failed to confirm such an association[6,7]. It is now widely recognized that clonogenic radiation sensitivity cannot fully explain the complex development of radiation injury, especially late damage. Radiation induces an orchestrated response cascade at both the cellular and tissue level. This response involves differential regulation of many cytokine cascades, which together have a profound impact on the resultant normal tissue damage.

There are now several studies in which microarray analyses have been applied to lymphoblastic or fibroblastic cells obtained from cancer patients undergoing

radiotherapy and irradiated *in vitro*. Early studies established that the technique could be used to identify sets of genes that were differentially expressed, in a dose-related fashion, at 1-3 d after ex-vivo irradiation[8,9]. Later studies assigned differentially expressed genes to functional groups, based on gene ontology (GO) terms in an attempt to identify pathways involved in the cellular response to ionizing radiation[1,10,11]. The majority of these studies did not attempt to link expression profiles of individual patients to the risk of radiation-induced damage, although some did investigate the relationship of expression profiles to known radiation sensitivity or cancer pre-disposition syndromes, like AT or BRCA1[12,13].

Only 4 studies have applied microarray analyses to compare expression profiles from patients with severe versus mild normal tissue damage after radiotherapy, in an attempt to build a predictive classifier. The first small study[14] used cytokine arrays from Atlas Clontech (with 268 transcripts spotted), to analyze base-line profiles of fibroblasts obtained from three breast cancer patients with minimal or no fibrosis after radiotherapy and three patients with severe fibrosis. All biopsies were taken from a site well away from the irradiation field, in order to investigate genetic factors rather than radiation induced factors relating to radiation sensitivity. Only minor differences between the two groups were found for expression levels of housekeeping genes, but 9 of the cytokine-receptor transcripts were differentially expressed between the severe and mild responders (subsequently confirmed by PCR analysis). Although interesting, this study was too small to generate a cytotoxic profile that could be used to predict patients at increased risk of development of late radiation fibrosis.

Another small clinical study was carried out in 5 head and neck cancer patients, to determine whether changes in gene expression in peripheral blood cells could be detected as patients progressed through a course of chemo-radiotherapy, and whether these changes could be related to the development of toxicity[15]. Expression profiles were generated from samples obtained before and two-weeks after the start of treatment. A database of genes over-expressed at 2 wk was created and their potential significance to toxicity was evaluated by canonical pathway analysis (using the Ingenuity Pathway Analysis library). Fourteen pathways, including inflammatory pathways like NFκB, IL-6 and VEGF signaling, were identified from analysis of circulating lymphocytes as being most relevant to the observed local toxicity. Although the sample size was small, the results from this study show that genetic changes induced by chemo-radiotherapy can be determined using peripheral blood cells and that these changes correlated with the clinical development of non-hematological, acute toxicity within the irradiation field.

In a larger study Rieger and colleagues used gene expression profiling after *ex vivo* irradiation to predict acute radiation toxicity in a diverse group of irradiated cancer patients[16]. They established lymphoblastoid cell lines from 14 patients who suffered severe acute toxicity and 13 patients with very mild toxicity and compared expression profiles at 4 h after 4 Gy. Significance Analysis of Microarrays (SAM) and nearest shrunken centroid (NSC) analyses were used to identify a set of 24 genes that were predictive for radiation toxicity. This classifier was able to identify 9 of the 14 over-responders (64%), with no false positives. Two of the patients with severe toxicity that were not identified were at risk from their aggressive treatment protocols and may well have been correctly assigned to the "normal response" group.

In a similar study, Svensson and colleagues used blood samples from 21 prostate cancer patients who developed severe late complications after radiotherapy and 17 patients without symptoms[17]. The lymphocytes were stimulated to divide *ex vivo* and irradiated (2 Gy) or sham treated (0 Gy) before analyzing expression profiles 24 h later. Profiles for radiation-induced genes were used to build a classifier for late radiation toxicity, based on expression levels of individual genes or sets of functionally or structurally related genes (using GO terminology). The classifier based on individual gene expression was able to correctly classify 63% patients (remarkably similar to the result obtained by Rieger and colleagues[16], whereas the gene set classifier was 86% accurate for the group as a whole. However, on an individual patient basis, only 55% patients could be correctly classified with a high degree of certainty.

These two studies clearly demonstrated a relationship between gene expression profiles for lymphocytes irradiated *ex vivo* and development of acute or late radiation injury. With further optimization such an approach may eventually be used to predict susceptibility to toxicity in individual cancer patients and identify those most at risk so that treatment schedules could be adjusted accordingly.

MICROARRAYS FOR UNRAVELLING MOLECULAR PATHWAYS INVOLVED IN ACUTE RADIATION TOXICITY, BEFORE THE ONSET OF TISSUE DAMAGE

A few experimental studies have used microarray analyses to study acute changes in gene expression during the first few days after *in vivo* irradiation of normal tissues *i.e.* before the onset of pathological tissue changes. In these studies, RNA extracts were made from whole tissue homogenates, not individual cell types as described in the above clinical studies. Whereas all were able to generate lists of differentially expressed genes, it was often difficult to extract biologically relevant information and most studies failed to identify new mechanistic pathways relating to the development of radiation injury in the tissues. The first such study evaluated overall gene expression profiles of irradiated mucosal tissue harvested from hamster cheek pouches at 1 h to 10 d after 35 Gy[18]. A group of 10 genes showed early upregulation (within 8 h), followed by delayed expression of other genes at later time points. Although some attempt was made to interpret the likely function of these genes in relation to the developing mucositis, no new mechanistic insights could be drawn from this study. There was also very little overlap between the microarray results and PCR data on a specific target group of genes.

Three experimental studies have used microarray analyses to investigate early changes in gene expression

in irradiated mouse brain. One study showed that very low doses of 0.1 and 2.0 Gy total body irradiation (TBI) induced expression of genes involved in protective repair functions and downregulated genes involved in neural signaling within 4 h[19]. Higher doses (2-20 Gy) were also associated with differential gene expression 5 h after whole brain irradiation[20]. Although there was very limited overlap between the genes identified in these two studies, different array platforms and analysis methods were used and it is difficult to draw general conclusions regarding mechanisms of early response to ionizing radiation in the brain. A third study showed early differential gene expression in both brain and kidneys at 8 and 24 h after TBI doses of 10 Gy. However, expression profiles were completely different in the two tissues, suggesting that tissue specific, rather than general stress response pathways may be activated[21].

MICROARRAYS FOR UNRAVELLING MOLECULAR PATHWAYS INVOLVED IN LATE RADIATION TOXICITY

There are only 5 publications, from 3 different groups, describing the use of microarrays to investigate mechanisms of delayed radiation fibrosis or vascular damage in normal tissues[22-26]. Johnston and colleagues compared expression profiles of irradiated (12.5 Gy) and sham treated (0 Gy) lung tissue, harvested from fibrosis sensitive or fibrosis resistant mice during the period of developing fibrosis (26 wk after thoracic irradiation)[22]. Several inflammatory chemokines and their receptors were upregulated in irradiated fibrosis prone mice but not in resistant strains. The increased expression of chemokines that are known to specifically recruit lymphocytes and macrophages, indicated that chronic inflammatory processes were probably involved in the fibrotic process in irradiated lungs of sensitive animals. Although 12K Affymetrix oligoarrays were used in this study, the authors only report expression levels of the CC chemokines and their receptors.

In our own studies, we used 15K cDNA microarrays in an attempt to identify time-related changes in gene expression in irradiated mouse kidney and rectal tissue, in relation to the onset and development of microvascular damage[23,24]. In the irradiated kidneys there was significant differential expression of a relatively small number of genes (< 0.5% spotted clones) during the period of development of telangiectasia (10-20 wk), with slightly more genes being downregulated at later times. In the rectum, a greater number (> 5%) of genes were differentially expressed at 10-20 wk after 20 Gy. Only five known genes and 14 ESTs that were upregulated during development of telangiectasia were common to both tissues. These included Jagged1 and KLF5 (Kruppel-like factor 5), which are both known to be involved in vascular development but had not previously been described in relation to radiation damage. Jagged1 is a ligand for the Notch receptor, which acts as a control gene that generally opposes the effects of growth factors stimulating angiogenic differentiation. It is overexpressed during repair of mechanical damage to large arteries and during the development of fibrotic disease[27,28]. Whether Jagged1 in irradiated kidney and rectal tissue is acting as a profibrotic stimulus, or whether it is directly involved in the development of telangiectasia, is not yet clear. Functional and histological studies after irradiation of genetically modified mice with reduced expression of Jagged1 may help to resolve this issue. KLF5 is normally downregulated in adult vessels but is overexpressed in response to vascular injury and it controls expression of growth factors like platelet derived growth factor and TGFβ[29]. It has not previously been linked with radiation injury. These microarray studies therefore identified new potential targets for intervention in the development of radiation induced vascular damage.

Only one group has published on the use of microarray technology to investigate changes in gene expression in relation to late radiation toxicity in cancer patients[25,26]. In these studies, gene expression from samples of healthy bowel were compared with samples from six patients with radiation enteritis (1-75 mo after radiotherapy), using either Clontech Atlas 1.2K cDNA arrays or extracellular matrix (ECM) specific pathway arrays (< 300 genes). Differential expression was found in 8% of genes studied. Samples from patients with radiation enteritis generally had increased expression of genes coding for proteins involved in composition and remodeling of the ECM (MMPs, TIMPs and collagens Ⅰ, Ⅲ, Ⅳ, Ⅵ and Ⅷ) and the Rho/Rock signaling pathway, which is implicated in tissue contraction and myofibroblast transdifferentiation. This increase was correlated with the degree of infiltration of the mucosa by inflammatory cells and the presence of differentiated mesanchymal cells in the submucosa. Genes involved in stress responses, antioxidant metabolism and inflammation, were also differentially expressed. The results from these studies suggest that radiation enteritis is a dynamic process, involving remodeling of structural components in the mucosa, with a balance towards fibrogenesis. Several new pathways (like Rho/Rock) were identified as participating in the response, in addition to confirming the involvement of known fibrotic pathways.

In summary, several experimental systems have been used for molecular profiling of radiation damage (including cell cultures, animal models and human samples) and some useful discoveries have been made. However, there are significant technical challenges associated with interpretation of expression profiling data and some obstacles remain to be overcome (see below).

HETEROGENEITY

Quantitative analysis of genes, while very informative, may not always be able to unravel the entire complexity of tissue damage processes *in vivo*. Radiation-induced normal tissue damage involves many different cell types and the composition of the tissue is likely to change as a result of radiation damage, especially at longer follow up times. There will, for example, be more inflammatory cells and more fibrotic tissue present after irradiation and cells from each population will be at various levels of activation, responding in different ways and to varying extents. The measured response will be a combination of multiple

factors, including gene expression changes in dying cells due to treatment and adaptive changes in surviving cells. As a consequence, changes in expression of relevant genes in target cells may be masked or diluted by the changes expression within the surrounding cells. Compensatory changes in other more abundant cell types may also negate any changes in the target cell. Thus, heterogeneity complicates the ability to draw conclusions about specific processes occurring within a tissue and interpretation of data in terms of mechanistic effects must therefore be done with caution when using material from whole tissue.

It should be noted, however, that some biological conditions can be understood only in the context of these heterogeneous cell populations[30]. For example, to investigate a process characterized by an inflammatory response, it must be understood that the inflammatory infiltrate is part of the process and thus part of the relevant difference in expression between irradiated and sham irradiated tissue. Identifying transcriptional responses using *in vitro* cell cultures may lead to spurious conclusions being drawn, since gene expression profiling may be limited to the model system employed, under specific conditions that may not necessarily extrapolate to different conditions or to the *in vivo* situation. Being able to measure gene expression profiles in individual target cells or cell types, by using laser capture microdissection (LCM) of tissues would be more desirable in this case[31,32]. In addition, verification using *in situ* hybridization of whole tissue samples would validate the results and provide spatial information.

Since proteins, as opposed to mRNA, represent the functional effectors of radiation induced normal tissue response and thus serve as therapeutic targets, integrating immunohistochemistry with gene expression approaches will provide additional insight into the most useful markers that warrant further investigation. It should be realized, however, that high quality antibodies are not always available for specific proteins of interest.

CROSS PLATFORM COMPARISON

While individual microarray studies can be highly informative, there are inconsistencies between various microarray platforms, making it almost impossible to compare independently obtained data sets addressing the same biological problem[33]. One of the difficulties in the cross-platform comparison of microarray data is to ascertain that probes on different platforms for the same gene do in fact quantify the same mRNA transcript. For the 6 years during which microarrays were available commercially, probe sequence information was not provided[34]. Users had to trust the manufacturer that a given probe actually quantified a specific transcript. In reality, many arrays used a substantial number of incorrect probes (wrong clones or misidentified ones) and a surprisingly large portion of the probes was not present in high-quality sequence databases such as RefSeq[35-40]. Apart from probe design issues, the discrepancies between an intended probe sequence and the actual sequence spotted on the microarray also deserve some attention. As our understanding of sequences and splice variants increased, probes have been eliminated or redesigned. Unfortunately, this had the undesirable side effect that data sets obtained on different generations of arrays could not be combined easily. This is much more of a problem for mechanistic than for profiling studies.

INTERPRETATION OF THE DATA

Data analysis and interpretation are essential elements in gene expression profiling and various approaches have been developed. Traditional strategies for gene expression analysis have focused on identifying genes that exhibit differences between two states of interest. Regardless of the methods used to select these genes (e.g. fold-changes or statistical significance), investigators are left with long lists of differentially expressed genes without knowing the biological relevance. This illustrates the large gap between our ability to collect data and to interpret the results. In response to this, some investigators try to attach some sort of conclusion to their microarray studies by selecting a few (known) genes for further investigation; this is more driven by the investigator's area of expertise than based on biological relevance. Moreover, the biological processes suggested to be involved often turn out to be so specific that they undermine the rationale for using global microarrays. Questions in which the expression of only one or a few genes, or a specific biological pathway, is concerned could be better addressed using other techniques, such as quantitative real time PCR or pathway-specific arrays.

Genome-wide profiling has moved well beyond the simple goal of identifying a few genes of interest and the common task is to translate lists of genes into a better understanding of the biological pathways involved. The work of the Gene Ontology (GO) Consortium provides a way to address this. The GO project (http://www.geneontology.org) is a collaborative effort to construct and use ontologies to facilitate the biologically meaningful annotations of genes and their products in a wide variety of organisms, by organizing genes into three key categories based on biological processes, molecular function and (sub) cellular localization[41-45]. The development of GOs allows investigators to look for trends in the data, by identifying changes in genes that share biological function.

The last few years have seen an explosion in the development of tools that can be used to derive biological relevance. The approach aims at establishing gene networks by integrating microarray data (analysis of differentially expressed genes) with array-independent analyses (e.g. literature references, protein interactions). Some applications that make use of this information and allow batch wise loading of the gene lists include FATIGO (http://fatigo.bioinfo.cnio.es[46]), GoMiner (http://discover.nci.nih.gov/gominer[47,48]) and the recent analysis software DAVID/EASE (http://david.abcc.ncifcrf.gov[49,50]). Each of these methods suffers from some limitation, since they fail to detect biological processes that are distributed across the entire network of genes and subtle changes at the level of individual genes. However, in spite of the limitations, this represents a much better approach than the manual retrieval of annotation on a gene-by-gene basis, which was the only

alternative in the past.

Assuming that gene expression changes can be better detected at the level of co-regulated genes rather than individual genes, Subramanian and colleagues devised a new user-friendly multiplatform software tool called Gene Set Enrichment Analysis (GSEA)[51,52]. Rather than focusing on individual genes (which can be poorly annotated and may not be reproducible), researchers focus on gene sets. These are defined based on prior biological knowledge e.g. published information on biochemical pathways or co expression in previous experiments. All genes can be ordered in a ranked list L, according to their differential expression between the classes. Given an *a priori* defined set of genes S (e.g. genes sharing the same GO category), the goal of GSEA is to determine whether the members of S are randomly distributed throughout the list L or primarily found at the top or bottom. We expect that sets related to the phenotypic distinction will tend to show the latter distribution.

GSEA features a number of advantages when compared with single gene methods. First, it considers all genes spotted on an array or in an experiment, not only those above an arbitrary cutoff in terms of fold-change or significance. It detects modest but coordinate changes in expression of groups of functionally related genes, and it facilitates the interpretation of large-scale experiments by identifying pathways and processes in an unbiased way. GSEA makes it easier to compare independently obtained datasets addressing the same biological problem, regardless of whether the same microarray platforms are used. Although subsequent analysis suggested that the statistical tools used in GSEA may be biased toward assigning higher enrichment scores to genes sets of large sizes[53], the program expands the potential for discovery of important process related genes in a give data set.

In conclusion, evaluation of microarray techniques indicates that they have advanced to a point where the question-driven profiling research has become a feasible complement to the conventional, hypothesis-driven research. Despite the limitations of data interpretation by understanding issues of tissue heterogeneity, and performing conformational studies in alternative platforms, gene expression profiling using microarrays represents a revolutionary technology. The limitations mentioned previously are not intended to discourage the application of gene expression profiling technologies to mechanistic or predictive assay development, but rather to guide experiments that will produce more useful and interpretable data in the near future.

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