Changes in Differential Gene Expression because of Warm Ischemia Time of Radical Prostatectomy Specimens

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The expression of thousands of genes can be monitored simultaneously using cDNA microarray technology. This technology is being used to understand the complexity of human disease. One significant technical concern regards potential alterations in gene expression because of the effect of tissue ischemia. This study evaluates the increase in the differential gene expression because of tissue processing time. To evaluate differential gene expression because of ischemia time, prostate samples were divided into five time points (0, 0.5, 1, 3, and 5 hours). Each time point consisted of a homogeneous mixture of 12 to 15 prostate tissue cubes (5 mm³). These tissues were maintained at room temperature until at the assigned time point the tissue was placed in OCT, flash frozen in liquid nitrogen, and stored at -80°C until RNA extraction. RNA from each time point was hybridized against an aliquot of 0 time point RNA from the same prostate. Four prostate glands were used in parallel studies. M-A plots were graphed to compare variability between time point sample hybridizations. Statistical Analysis of Microarray software was used to identify genes overexpressed at the 1-hour time point versus the 0-hour time with statistical significance. Microarray analysis revealed only a small percentage of genes (<0.6%) from more than 9000 to demonstrate overexpression at the 1-hour time point. Among the 41 statistically significant named overexpressed genes at the 1-hour time point were early growth response 1 (EGR1), jun B proto-oncogene (jun B), jun D proto-oncogene (jun D), and activating transcription factor 3 (ATF3). Genes previously associated with prostate cancer did not have significantly altered expression with ischemia time. Increased EGR1 protein expression was confirmed by Western blot analysis. Microarray technology has opened the possibility of evaluating the expression of a multitude of genes simultaneously, however, the interpretation of this complex data needs to be assessed circumspectly using refined statistical methods. Because RNA ex-

pression represents the tissue response to insults such as ischemia, and is also sensitive to degradation, investigators need be mindful of confounding artifacts secondary to tissue processing. All attempts should be made to process tissue rapidly to ensure that the microarray gene profile accurately represents the state of the cells and confirmatory studies should be performed using alternative methods (eg, Northern blot analysis, Western blot, immunohistochemistry). (*Am J Pathol* 2002, 161:1743-1748)

Genome-wide scans of cancer samples has been facilitated by the use of cDNA microarray technology. This technology provides a unique method of evaluating the differential gene expression of several thousand genes simultaneously.^{1,2} In cancer molecular biology this technique has been used not only to identify genes involved in tumorigenesis, but also to identify genes that may be clinically useful molecular markers. Several groups including our own have recently applied cDNA microarray technology as a tool to study prostate cancer with the goal of identifying clinically significant markers of biologically aggressive disease.³⁻⁷ Because the technique of cDNA microarray analysis generates complex data sets, the conclusions derived are greatly dependent on the methods of data interpretation. In addition, technical concerns arise as well particularly with respect to the processing of tissue used for such experiments.

One specific area of concern is the effect of ischemia on the profile of differential gene expression. Microarray technology has identified some putative prostate cancer biomarkers such as hepsin and α -methylacyl-CoA racemase (AMACR).^{3,4,8,9} However other putative candidates need to be further characterized. In this process it is important to exclude spuriously deregulated genes because of such technical artifacts such as prolonged warm ischemia before processing. This may be a more

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important issue within the early time period after surgical extirpation when the cellular metabolic machinery can mount a survival or apoptotic response before all metabolic activity ceases. Conversely genes that appear down-regulated could represent an artifact of RNA degradation with prolonged warm ischemia time. Some genes may also have greater susceptibility to degradation. Negative expression, therefore, is more difficult to ascertain because there is no way to distinguish decreased expression from degradation. Thus we proposed to study the increase in the differential gene expression profile throughout time of prostate tissue obtained from radical prostatectomy specimens removed as treatment for localized prostate cancer and identify individual genes that may be artifacts of processing.

Materials and Methods

With Institutional Review Board approval, four radical prostatectomy specimens were collected at the time of surgical removal and transported within 5 minutes to the surgical pathology laboratory for immediate processing. Time courses from each prostate specimen were performed independently and treated as individual experiments. Excess tissue from the clinical pathology sample was fractionated into 0-, 0.5-, 1-, 3-, and 5-hour time points. Each time point consisted of a mixture of 12 to 15 prostate tissue cubes (5 mm³) from throughout the entire prostate specimen. This was done to assure that the samples contained a homogenous mixture of stroma and epithelial cells, and benign and cancerous cells from the same prostate. These tissues were maintained at room temperature until the assigned time point and the tissue was placed in OCT, flash frozen in liquid nitrogen, and stored at -80°C until RNA extraction. In Trizol reagent (Life Technologies, Inc., Rockville, MD) tissue from the five time points were separately homogenized. RNA was obtained using a standard phenol/chloroform extraction. The integrity of total RNA was confirmed by denaturing formaldehyde agarose gel electrophoresis for each of the time points. The RNA was evaluated by spectrophotometry, qualitatively by the A_{260/280} ratio, and quantitatively by the absorbance at 280 nm.

The spotted glass cDNA microarray slides used in this study included ~5000 known, named genes from the Research Genetics human cDNA clone set, 4400 expressed sequence tags.³ Fluorescently labeled (Cy5) cDNA was prepared from total RNA from each of the ischemia time points. The reference zero (0 hour) samples used in this study were labeled using a second distinguishable fluorescent dye (Cy3) using a previously established protocol (www.microarrays.org). After labeling, the cDNA samples were neutralized, washed, and then applied to the microarray chips. After remaining in a hybridization water bath at 65°C overnight, the microarray slides were processed and scanned with a Genepix 4000 scanner.

Primary analysis was done using the Genepix software package. Images of scanned microarrays were gridded and linked to a gene print list. Initially, data were viewed

as a scatter plot of Cy3 *versus* Cy5 intensities. Cy3 to Cy5 ratios are determined for the individual genes along with various other quality-control parameters (eg, intensity over local background). The Genepix software analysis package flags spots as absent based on spot characteristics. Furthermore, bad spots or areas of the array with obvious defects were manually flagged. Spots with small diameters (<50 μm) and spots with low signal strengths <350 fluorescence intensity units over local background in the more intense channel were discarded. Flagged spots were not included in subsequent analyses. Data are the ratio of the fluorescent cDNA probe signal hybridized against each time point to the reference 0-hour time point including the 0-hour time point itself.

Analysis introduced by ischemia time used a method, previously described, in comparing microarray quality that we have used to compare experimental samples.^{10,11} Briefly, we created a M-A plot where $M = \log(Cy5/Cy3)$ is the log ratio of the two dyes used in the hybridization, and $A = [\log(Cy5) + \log(Cy3)]/2$ is the average of the log intensities. In our experiments, the Cy5-labeled time point and the Cy3-labeled 0-time reference were hybridized together. The M-A plot of the 1-hour point hybridization (1-hour Cy5 labeled *versus* 0-hour Cy3 labeled) is compared to the 0-time point hybridization (Cy5 labeled 0 hour *versus* 0-hour Cy3 labeled). We summarized the intensity data by taking the averaging Cy3 and Cy5 intensities for all of the genes for the four time courses at the 0-hour and 1-hour time points separately.

To identify statistically significant genes induced by ischemia, we analyzed our data from the four time courses with Statistical Analysis of Microarray (SAM) software (Stanford University, Stanford, CA), as previously described.¹² Briefly, SAM performs repetitive permutations based on gene-specific *t*-tests to identify statistically significant genes. Adjustment of a delta-tuning parameter can alter the number of false-positives, and use of threshold parameters instructs that magnitude of expression change meets cutoff requirements. Only over-expressed genes were analyzed because of the potential confounding effect of RNA degradation contributing to the number of underexpressed genes. In this experiment we used a delta of 0.20, upper threshold of 2.5-fold overexpression, and 0.4-fold under expression using a one class comparison at the 1-hour time point, because the reference in each hybridization was the 0-hour time point. The 1-hour time point was chosen because most surgical specimens are realistically processed within this time frame. Some of the genes of interest identified by SAM along with genes involved in prostate cancer were plotted together as a heat map using the Cluster/Tree-View program (Stanford University).

Western blot analysis of time course was performed for one of the genes of interest, early growth response 1 (EGR1), with a readily available commercial antibody. The Western blot was performed as a time course from a single one of the prostates used in the cDNA expression microarray at the same time points. Tissues were homogenized in Nonidet P-40 lysis buffer containing 50 mmol/L Tris-HCl, pH 7.4, 1% Nonidet P-40 (Sigma, St. Louis, MO), and complete proteinase inhibitor cocktail tablet

Figure 1. M-A plots at the 0- and 1-hour time points. M-A plot where $M = \log(\text{Cy5}/\text{Cy3})$ is the log ratio of the two dyes used in the hybridization, and $A = [\log(\text{Cy5}) + \log(\text{Cy3})]/2$ is the average of the log intensities. **A:** The Cy5-labeled time point and the Cy3-labeled 0-time reference were hybridized together. **B:** The M-A plot of the 1-hour point hybridization (1-hour Cy5 labeled *versus* 0-hour Cy3 labeled) is compared to the 0 time point hybridization (Cy5 labeled 0 hour *versus* 0-hour Cy3 labeled). The data are summarized by taking the average Cy3 and Cy5 intensities for all of the genes for the four time courses at the 0-hour and 1-hour time points separately.

(Roche, Indianapolis, IN). After quantification using a standard Bradford assay, 15 μg of tissue protein extracts were mixed with sodium dodecyl sulfate sample buffer and electrophoresed onto a 10% sodium dodecyl sulfate-polyacrylamide gel under reducing conditions. The separated proteins were transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was incubated for 1 hour in blocking buffer [Tris-buffered saline with 0.1% Tween (TBS-T) with 5% non-fat dry milk]. The EGR1 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) was applied at 1:1000 dilution in blocking buffer overnight at 4°C. After washing three times with TBS-T buffer, the membrane was incubated with horseradish peroxidase-linked donkey anti-rabbit IgG antibody (Amersham Pharmacia Biotech) at 1:5000 for 1 hour at room temperature. The signals were visualized with the chemiluminescence enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) and autoradiography. For β -tubulin control, the EGR1 antibody probed membrane was stripped with Western Re-Probe buffer (Geno-tech, St. Louis, MO) and blocked in TBS-T with 5% nonfat dry milk and incubated with rabbit anti- β -tubulin antibodies (Santa Cruz Biotechnologies) at 1:500 for 2 hours. Visualization was performed after application of the secondary antibody as described above.

Results

Hybridization at five time points (0, 0.5, 1, 3, and 5 hours) yielded results for each of the four prostate time courses. M-A plots from the 0- and 1-hour time points visually depict little overall variation between samples (Figure 1). The SAM analysis identified 61 statistically significant genes that were overexpressed at the 1-hour time point. This represents only a small percentage of genes (<0.6%) with overexpression at the 1-hour time point. The

41 significant named genes are presented in Table 1. At least several of these genes are known to be early response genes, genes implicated in hypoxia, or transcription factors, including EGR1,¹³ jun B proto-oncogene (JUNB),¹⁴ jun D proto-oncogene (JUND),¹⁵ and activating transcription factor 3 (ATF3).¹⁶ The expression of these genes for the four time courses throughout five time points are compared in a heat map to genes implicated in prostate cancer, such as hepsin,³ AMACR,^{8,9} fatty acid synthase, PTEN, and PIM-1³ (Figure 2). The gene expression for this group of cancer-related genes remains relatively constant throughout the four time courses as demonstrated by their minimal degree of red color change through the four time courses.

We analyzed four of the above genes in greater detail. The means of the time point expression ratios (each time point hybridized against 0 time) for all four prostate time courses was plotted for two of the genes identified as significant by the SAM analysis, EGR1 and jun B (Figure 3A). The gene expression throughout time for these two genes increases to a steady level by 3 hours. Whereas, the expression of two genes implicated as prostate cancer markers, hepsin and AMACR, remains steady throughout the time course (Figure 3B).

Confirmatory Western blot analysis with an anti-EGR1 antibody is presented in Figure 4, where each lane corresponds to a point in the time course. A band at 58 kd corresponds to the expected weight of the 543-amino acid-long EGR1 protein. The intensity of the bands increases throughout the time course, implying increased EGR1 protein expression with time.

Discussion

cDNA microarray technology has become a widely used tool for molecular profiling. The techniques used to ana-

Table 1. Statistically Significant Named Genes with Overexpression at 1-Hour Time Point

Gene name	Gene ID
ZFP36, zinc finger protein 36, C3H type, homolog (mouse)	23804
CRYAB, crystalline, alpha B	839763
TTC1, tetratricopeptide repeat domain 1	725274
EGR1, early growth response 1	840944
JUNB, jun B proto-oncogene	309864
MT1L -OR- MT1X, metallothionein 1L -OR- metallothionein 1X	297392
USP14, ubiquitin specific protease 14 (tRNA-guanine transglycosylase)	81599
ZNF76, zinc finger protein 76 (expressed in testis)	745003
MYL2, myosin, light polypeptide 2, regulatory, cardiac, slow	300051
EGR1 -OR- DCT, early growth response 1 -OR- dopachrome tautomerase	753104
AGXT, alanine-glyoxylate aminotransferase	247117
JUND, jun D proto-oncogene	767784
ACADVL, acyl-coenzyme A dehydrogenase, very long chain	810358
BTG2, BTG family, member 2	213136
SPOCK, sparc/osteonectin, cwcv and kazal-like domains proteoglycan	754358
BLVRB, biliverdin reductase B	246035
HNRPA1, heterogeneous nuclear ribonucleoprotein A1	511586
DDM36, DDM36	284101
ETV5, ets variant gene 5 (ets-related molecule)	796542
H2BFQ, H2B histone family, member Q	813149
UROS, uroporphyrinogen III synthase (congenital erythropoietic porphyria)	809454
HCA127, hepatocellular carcinoma-associated antigen 127	358549
ASAH1, N-acylsphingosine amidohydrolase (acid ceramidase)-like	324342
SLC7A6, solute carrier family 7	767769
APOC2, apolipoprotein C-II	809523
CISH, cytokine inducible SH2-containing protein	771058
EIF2S3, eukaryotic translation initiation factor 2, subunit 3 (gamma, 52kD)	784841
MRPL12, mitochondrial ribosomal protein L12	810173
PPAP2B, phosphatidic acid phosphatase type 2B	85394
MINK, misshapen/NIK-related kinase	756211
VTN, vitronectin	246073
MB, myoglobin	611443
ATF3, activating transcription factor 3	51448
MGC4730, similar to RIKEN cDNA 3110001D03 gene (M. musculus)	359269
HGF, hepatocyte growth factor (hepapoietin A; scatter factor)	41650
VCAM1, vascular cell adhesion molecule 1	49164
JUN, v-jun sarcoma virus 17 oncogene homolog (avian)	358531
NUP88, nucleoporin 88kD	843070
C21orf11, chromosome 21 open reading frame 11	186918
PCYT2, phosphate cytidylyltransferase 2, ethanolamine	130884
RALY, RNA binding protein	825583

lyze the vast quantity of data generated are variable, but systematic statistical methods for analyzing the data and accounting for the inherent variability of the experiment (microarray chip quality and differential labeling) are being introduced. Little research thus far has been devoted to systemic variability contributed by the samples themselves beyond the heterogeneity involved in using biological tissue.

To our knowledge, Huang and colleagues¹⁷ were the first to study the effects of ischemia on the gene expression profile probed by cDNA microarrays. These investigators were interested in the general pattern of expression in colon cancer changes secondary to the ischemic effect. They used a latent class model to observe a change from the average gene expression. This study preliminarily established that ischemia alters the gene expression profile in tissue, but was severely limited by analyzing only one specimen and using a cell line as the reference.

Our study is the first to introduce the concept of systemic sample effect. In our experiments this effect was because of the ischemia time of tissue processing. By performing our time-course experiments independently on four samples, we have mitigated the problem of false-positives introduced by a lack of replicates to identify a group of genes that are overexpressed in prostate tissue solely because of ischemia.¹⁸ For at least one of these genes, EGR1, we were also able to demonstrate increased protein expression throughout time.

Our study presumed that there would be altered gene expression throughout time; interestingly however, there was little overall increase in the gene expression variability present with ischemia time, as depicted in the M-A plots. We assumed that we would not be able to distinguish degradation from decreased expression; therefore we pursued genes with a pattern of expression that peaked at 1 hour. In our radical prostatectomy harvesting protocol, most surgical specimens are routinely processed within 30 minutes after surgical extirpation. However, keeping in mind the logistics of transporting the freshly extirpated specimen, and accessioning, examining, and sectioning the specimen, we believed that 1 hour would be a realistic time frame before the specimen can be frozen to prevent further RNA degradation. With this time course in mind, we identified four reference genes: EGR1, Jun B, Jun D, and ATF3 that increased in expression throughout time. The longer time points were examined as extreme examples for radical prostatectomy samples. However, in some of our prostate cancer-profiling work, we have used advanced tumor from a rapid autopsy program, in which samples may be harvested up to 3 to 5 hours after death.¹⁹

Several studies have now begun to exploit the high-throughput capabilities of cDNA microarray to obtain a molecular profile of prostate cancer.^{3-7,20} Several interesting genes that play a putative role in prostate cancer have been identified. These notably include two genes that have aroused interest recently, hepsin³⁻⁶ and AMACR.^{8,9} The usefulness of the present study is to evaluate whether the processing time may influence the gene expression profile for prostate tissue specimens. Although we identified several genes with a statistically significant increase in expression at 1 hour, none of the recently reported genes involved in prostate cancer development appeared to be dramatically affected by ischemia time.

Of the 41 significant named genes that met our criteria for overexpression at 1 hour, several have been identified in the literature for increased expression secondary to

Figure 2. TreeView analysis of benign prostate tissue demonstrating differential gene expression at different time intervals because of warm ischemia. Overexpressed and underexpressed genes are represented in red and green, respectively. Black represents no change in expression. Color intensity corresponds to the degree of overexpression or underexpression with brighter shading indicating higher level of expression.

ischemic stress. Jun B and jun D transcript levels are elevated after cerebral ischemia in animal models.^{14,15} Activating transcription factor 3 (ATF3) is induced by ischemia in a variety of tissues including heart, liver, kidney, and brain.¹⁶ In most tissue types ATF3 mRNA expression is increased within 2 hours of the insult.¹⁶ EGR1 appears to function as a master switch to activate several cellular responses to ischemic stress rapidly after the insult.¹³ Most of the overexpressed genes we identified were not related to prostate cancer; except for one of these genes, EGR1.^{21,22} Eid and colleagues²¹ compared EGR1 expression in prostate cancer from transurethral prostatectomy and total prostatectomy specimens from men with untreated disease *versus* normal prostate obtained during autopsy of organ donors. EGR1 mRNA expression was elevated in prostate cancer by Northern blot analysis. *In situ* hybridization confirmed the finding and showed expression mainly confined to the epithe-

lium. Quantitative *in situ* hybridization revealed increased EGR1 expression with higher Gleason grade cancer. Finally EGR1 protein was confined to the epithelium by immunohistochemistry, a finding confirmed by others.²² In our study, in which the relative expression of all genes were compared to their 0-hour time point for all time points, we found EGR1 to be exemplary of a gene with increased expression with ischemia time. In contrast, the relative expression of hepsin, a gene whose overexpression has been confirmed in several studies and been validated by Northern blot analysis and immunohistochemistry,³ remained constant throughout the time course. That two genes previously identified with prostate cancer could have different expression patterns with ischemia time would imply that some prostate cancer genes might be more susceptible to ischemia. EGR1 in particular may be such a gene because of its central role in the cellular response to ischemia.¹³ Alternatively, increased gene expression is the artifact of tissue processing that could have begun with tissue ischemia while ligating the blood supply to the prostate during surgery that continued until freezing, although this source of error should have been minimized by using a 0-time point as the reference during hybridizations.

Based on the work of Huang and colleagues²³ and the current study, confirmation of differentially expressed genes by cDNA analysis is critical. In our study a pattern of gene expression including EGR1, jun B, jun D, and ATF 3 suggests an ischemia effect. We recommend analyzing prostate cancer cDNA microarray data circumspectly if this pattern is present. Furthermore, in this study we validated EGR1 expression with Western blot analysis, but various other confirmation techniques are available and should be used before overstating the potential role of a biomarker.

Figure 3. cDNA microarray gene expression intensity of select genes throughout time course. **A:** Genes such as EGR-1 and jun-b demonstrated near twofold increase throughout time because of ischemia. **B:** Other genes previously reported as putative prostate cancer biomarkers demonstrated little change throughout the same time course.

Figure 4. Western blot analysis with anti-EGR1 antibody throughout the time course (0, 0.5, 1, 3, and 5 hours) confirms increased EGR-1 protein accumulation throughout time, confirming the expression array data. β-tubulin serves as a control for protein loading.

Conclusion

Microarray technology is a tool that has opened the possibility of evaluating the expression of a multitude of genes simultaneously, however, the interpretation of this complex data needs to be assessed circumspectly using refined statistical methods. Because RNA expression represents the tissue response to insults such as ischemia, and is also sensitive to degradation, we further

caution investigators to be mindful of confounding artifacts secondary to tissue processing. All attempts should be made to process tissue rapidly to ensure that the microarray gene profile accurately represents the state of the cells. Confirmation should also be sought using alternative methods (eg, Northern blot analysis, Western blot, immunohistochemistry).

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