Application of Oligonucleotide Microarrays to Assess the Biological Effects of Neoadjuvant Imatinib Mesylate Treatment for Localized Prostate Cancer

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Abstract Purpose: Neoadjuvant administration of antineoplastic therapies is used to rapidly assess the clinical and biological activity of novel systemic treatments. To assess the feasibility of using microarrays to assess molecular end points following targeted treatment in a heterogeneous tumor, we measured global gene expression in localized prostate cancer before and following neoadjuvant treatment with imatinib mesylate.

Patients and Methods: Patients with intermediate-risk to high-risk prostate cancer were treated for 6 weeks with 200 to 300 mg of oral imatinib mesylate. Frozen tissue was obtained from pretreatment ultrasound-guided biopsies and posttreatment radical prostatectomy specimens. Oligonucleotide microarray analysis following laser capture microdissection (LCM) and RNA amplification was used to assess gene expression changes associated with imatinib mesylate therapy. Immunohistochemistry was used to measure protein expression of MKP1 and CD31 and to assess cellular apoptosis.

Results: Of the 11 patients enrolled, high-quality microarray data was obtained from both biopsies (n = 7) and radical prostatectomy specimens (n = 9). Technically introduced intrasample gene expression variability was found to be significantly less than intertumor biological variability. Large gene expression differences were observed, and the gene with the most consistent differential expression (MKP1) was validated by immunohistochemistry. Gene set enrichment analysis suggests that imatinib mesylate therapy results in apoptosis of microvascular endothelial cells, an observation anecdotally supported by immunohistochemistry.

Conclusions: This study shows that high-quality microarray data can be generated using LCM and RNA amplification to discover potential mechanisms of targeted therapy in cancer.

Neoadjuvant therapy for cancer can reduce tumor burden, treat early metastatic disease, and allow an assessment of tumor sensitivity (1–6). Eradication of local disease in response to neoadjuvant therapy predicts improved recurrence-free and disease-specific survival (7–9). In addition, neoadjuvant therapy can enable a biological assessment of tumor responses to established and/or novel treatments (10–12). However, as many treatments lack a robust molecular readout of drug effect and the precise mechanisms of antitumor agents remain unknown, the application of high-information content, unbiased assays has the potential to identify markers of drug efficacy and elucidate mechanisms of action.

DNA microarrays have enabled significant discovery in clinical and molecular oncology. Investigators have applied microarrays to identify diagnostic markers (13–15), prognostic markers (15, 16), and molecularly distinct subclasses of tumors (17–19). Additionally, microarrays have identified multigene models predicting recurrence following surgery (20, 21), disease-specific mortality (22, 23), and chemotherapy sensitivity (24–27). The broad application of this powerful technology, including neoadjuvant trials, however, has been limited because of the relatively large amount of tissue required for analysis.

To perform microarray analysis on small tumor specimens (i.e., biopsies) and minute quantities of RNA, investigators have modified standard methods of RNA processing so as to include increased RNA amplification (28). In general, two serial
rounds of cDNA synthesis and in vitro transcription are used to amplify the RNA and obtain a sufficient yield of labeled product for hybridization to microarrays. This approach facilitates microarray analysis of small samples feasibly obtained in the neoadjuvant setting. The incorporation of laser capture microdissection (LCM) enables investigators to focus on specific cell populations (13). Although these methods have become more established, the feasibility of applying LCM and RNA amplification to identify global gene expression differences in the setting of neoadjuvant treatment has not been clearly shown.

Prostate cancer is the most commonly diagnosed non-dermatologic cancer in North American men, and ~200,000 men will be diagnosed in 2005 (ACS data). Although DNA microarrays have enabled significant discovery in prostate cancer (13–16, 20, 21), the small volume of prostate cancer at time of diagnosis challenges the application of this powerful technology.

In this report, we adapt the most commonly used method of RNA amplification and apply it to clinical samples from a neoadjuvant trial testing imatinib mesylate in prostate cancer. We find that global gene expression following RNA amplification is feasible from frozen cancer specimens obtained by biopsy before treatment and from radical prostatectomy specimens following treatment. The most differentially expressed gene identified during supervised analysis was validated by immunohistochemistry, and global gene expression changes suggest that imatinib mesylate may target the cancer associated vasculature.

Materials and Methods

Sample collection. We obtained frozen tumors for microarray analysis from two sources. Localized prostate cancer specimens before and following STI571 treatment were obtained from men with prostate cancer enrolled on Dana-Farber Cancer Institute protocol #01-172 entitled “An open-label, pilot study of orally administered STI571 before radical prostatectomy in patients with newly diagnosed prostate.” And, as additional controls, five anonymous, untreated prostate cancer specimens were obtained from men enrolled on the Dana-Farber Cancer Institute protocol # 01-045 entitled “Collection of specimens and clinical data for patients with prostate cancer or at high risk for prostate cancer.”

All samples were collected and processed rapidly to minimize RNA degradation, embedded in optimal cutting temperature compound, and stored in liquid nitrogen. Prostate biopsy specimens (pretreatment tissue for participants in Dana-Farber Cancer Institute #01-172) were immediately frozen on premade, frozen pallets of optimal cutting temperature compound, covered with additional optimal cutting temperature compound, and immersed in liquid nitrogen. Localized prostate cancer specimens from radical prostatectomy specimens were collected during gross pathologic evaluation of the ex vivo prostate immediately after removal. Prostates were sectioned from apex to base every ~0.5 cm and investigated for regions of tumor. Samples were taken from suspicious areas, embedded in optimal cutting temperature, and frozen in liquid nitrogen. A pathologist expert in prostate cancer (M.L.) evaluated all samples histologically before processing for microarray analysis.

Treatment protocol. The eligibility criteria for the Dana-Farber Cancer Institute protocol #01-172 included men who were good surgical candidates and diagnosed with intermediate-risk to high-risk adenocarcinoma of the prostate (see Supplementary Methods for protocol details). Patients were screened for the study with a physical exam, basic laboratory chemistry, serum prostate-specific antigen (PSA), and pathologic review. Once enrolled, patients had an initial evaluation, including prostate imaging (magnetic resonance imaging/magnetic resonance spectroscopy and positron emission tomography scanning), serum PSA and vascular endothelial growth factor levels, and ultrasound-guided biopsies. The patients were treated with 6 weeks of imatinib mesylate at 200 or 300 mg orally twice a day, during which time they had biweekly physical exams to assess for toxicity and compliance with the medications. After completing therapy, patients underwent repeated imaging and serum studies and subsequently had a radical prostatectomy. There was no gap between imatinib therapy and surgery; patients continued to take imatinib until surgery, including taking a tablet on the morning of surgery. Along with standard pathologic evaluation, patients’ samples were stained for CD31, Ki67, and Apoptag.

LCM. Eight-micrometer-thick frozen sections of each specimen containing prostate cancer was cut and stained with H&E using a slightly modified previously described protocol (ref. 29; see Supplementary Methods for details). LCM was done on sections using a PixCell II LCM System (Arcturus Engineering, Mountain View, CA) and previously described methodology (30). Specifically, ~4,000 laser pulses of 15-μm diameter and 25 mW pulse power were used to collect malignant epithelial cells. For biopsy samples with scant tumor tissue, multiple sections were used to reach a total of 4,000 laser pulses. Immediately following LCM, captured tissue was dissolved in lysis buffer from the Absolutely RNA Nanoprep Kit (Stratagene, La Jolla, CA) and frozen at −20 C until RNA isolation. Representative images before and after LCM were obtained for each sample.

Target preparation and microarray hybridization. Total RNA was isolated from samples following LCM using the Absolutely RNA Nanoprep Kit (Stratagene) and eluted into a final volume of 10 μL as described by the manufacturer. The 10 μL of eluant was used in its entirety to begin RNA amplification. All samples were processed using two rounds of cDNA synthesis followed by in vitro transcription with a modified protocol created by adapting previously published methods (refs. 28, 31–34; see Supplemental Methods for details).

Fifteen micrograms of biotin-labeled cRNA were fragmented and hybridized to U133A microarrays (Affymetrix, Santa Clara, CA), and the microarrays were subsequently scanned all using previously described methods (18, 20, 31). Gene expression was measured using MAS GeneChip software (Affymetrix). Samples from the STI571 clinical trial were processed in multiple batches. Pretreatment and posttreatment samples were processed together for paired specimens. Positive and negative controls were processed in each batch to assess the success of RNA amplification.

Expression analysis. Each sample microarray file was assessed for overall quality based on array intensity, mean gene expression value, and the percentage of genes called present. Hierarchical clustering was used to organize all samples based on gene expression alone using dChip software (ref. 18; see Supplementary Methods for details). Supervised analysis was done to identify genes with expression changes in response to imatinib mesylate. Gene expression in pretreatment biopsy samples (n = 7) and untreated prostatectomy specimens (n = 3) was compared with that in prostatectomy specimens following treatment with imatinib mesylate (n = 9) using a signal-to-noise score (31). The statistical significance of the measured difference in expression was determined using permutation testing (20, 31). Two-tailed Mann-Whitney tests were used to compare correlations between replicates and samples using Prism software (version 4.02, GraphPad Software, Inc., San Diego, CA).

To identify pathways potentially altered in prostate cancer in response to imatinib mesylate therapy, gene set enrichment analysis (GSEA) was done (35, 36). Here, a curated list of 540 pathways and their associated genes (37) was used to determine if any gene set(s) had coordinate differential expression greater than expected by chance alone. A nominally P ≤ 0.05 was taken as statistically significant and used to rank pathways along with the calculated false detection rate for each pathway.
**Immunohistochemistry.** Sections of 4-μm-thick, paraffin-embedded slides were dewaxed and rehydrated using xylene and ethanol, respectively. The MKP1 was done as previously described (38, 39). Briefly, antibody was incubated overnight in a 1:200 dilution at 4°C. The secondary antibody was biotin labeled and was applied for 30 minutes. Streptavidin-LSA amplification method (DAKO K0679, Carpinteria, CA) was carried out for 30 minutes followed by peroxidase/diaminobenzidine substrate/chromagen. The slides were counterstained with hematoxylin. The intensity of the brown chromogen was determined using a semiautomated image analysis system (Chromavision Medical Systems, Inc., San Juan Capistrano, CA) ACIS II. Briefly, ACIS II consists of a microscope with a computer-controlled mechanical stage. Proprietary software is used to detect the brown stain intensity of the chromogen used for the immunohistochemical analysis and compares this value to blue counter stain used as background. Theoretical intensity levels range from 0 to 255 arbitrary units. To ensure that the staining intensity was read only from neoplastic cells and not adjacent benign glands, the study pathologist circled only areas of histologically recognizable prostate cancer.

**Results**

**Patient accrual, response, and specimen collection.** To determine the feasibility of using microarray analysis to assess imatinib mesylate treatment in prostate cancer, we sought to collect tumor before and following treatment. Obtaining frozen prostate tissue with cancer cells before and after treatment presents a particular challenge; biopsies are uncomfortable for patients and may not contain tumor due to the low volume disease generally diagnosed in the era of PSA screening and poor radiological visualization. In the end, 11 of a planned 19 patients enrolled and received biopsies, treatment with imatinib mesylate, and radical prostatectomy (Fig. 1A).

No consistent trend in serum PSA levels during therapy was observed; three patients had a decrease in PSA of >25%, four patients had a relatively stable PSA (change of <25% in either direction), and four patients had an increase in PSA of >25% (Table 1). Prostate cancer was present in all 11 paraffin-embedded prostate specimens, and no consistent change in pathology (i.e., Gleason sum) was observed.

In this group of patients with intermediate-risk (n = 10) or high-risk (n = 1) disease, a relatively high proportion of the biopsies and radical prostatectomy specimens contained tumor. Of the 11 patients enrolled, prostate cancer was present in 7 (64%) of the ultrasound-guided biopsies and 9 (82%) of the frozen tissue from the radical prostatectomy specimen. This compares very favorably to that of our previously established tumor bank of 235 samples collected from radical prostatectomy specimens, where only 65 (28%) collected as “tumor” contained sufficient cancer for analysis (20).

**Technical versus biological variation.** LCM collection of cancer cells from biopsies and prostatectomy specimens significantly changes the amount of total RNA feasibly collected. Although our standard protocols require 5 to 15 μg of total RNA (20, 31), our RNA yields are ~50 ng with 4,000 laser pulses. Consequently, new methods had to be adapted for this study. However, LCM and RNA amplification may introduce obfuscating variation in gene expression because of the increased sample handling and additional enzymatic processes compared with our standard methods. To determine the extent of expression artifact introduced by LCM and/or RNA amplification and the reproducibility of the expression data, batch controls using diluted cell line RNA and multiple replicates samples were collected and analyzed during sample processing.

All samples were processed with our standardized protocol, including LCM to collect malignant epithelial cells, RNA amplification, and microarray hybridization (U133A microarrays, Affymetrix) to minimize differences between biopsy and radical prostatectomy specimens (Fig. 1B). The median Pearson correlation coefficient between controls from any two batches was 0.95 (Fig. 2A). A single control (PC4) had consistently lower correlation with all other controls (mean = 0.87), suggesting a greater degree of technically introduced variation in gene expression that may also affect experimental samples processed with PC4.
Interpreting the significance of the observed degree of correlation requires an understanding of the correlation between replicate tumor samples and between different tumors. The mean correlation between replicate tumor samples was 0.961 ± 0.022 (n = 8 pairs of replicates). Here, a replicate samples denote separate LCM collection of cells, RNA isolation, and RNA amplification from adjacent sections of the same tumor. Although there is unlikely to be significant biological difference between collected cells given the limited size of the biopsies and specimens from radical prostatectomy, some biological variation could contribute to the observed differences. Regardless, the correlation between tumors (0.630 ± 0.150) was significantly less than between control samples across batches (P < 0.001) or replicate samples (P ≤ 0.001; Fig. 2B). There was not a significant difference between the mean correlation of controls and replicates (P = 0.087). Thus, whereas LCM and RNA amplification clearly contribute some technical variation to gene expression, the degree of technically introduced variation seems to be much less than the variation between tumors.

Unsupervised analysis using hierarchical clusters further supports our observation that technically introduced gene variation is relatively small compared with gene variation between tumors. When samples are organized based on gene expression alone using Cluster software (Eisen Lab), sample identity was the primary organizing feature of all samples.

Table 1. Patient characteristics

<table>
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<th>Patient no.</th>
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<th>Posttreatment data</th>
<th>Biochemical and pathologic response</th>
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<td>Gleason score *</td>
<td>Posttreatment PSA</td>
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<td>4 + 3 = 7</td>
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</table>

*All biopsies and radical prostatectomy specimens were reviewed by pathologists at Brigham and Women’s Hospital.

Fig. 2. Correlation of gene expression. A, Pearson correlation coefficient for the five control cell line RNA run with each batch of samples. B, box plots of Pearson coefficient distributions for cell line controls, tumor sample replicates, and nonreplicate tumors. *, P < 0.00001; **, P < 0.0001, two-tailed Mann-Whitney test. C, hierarchical cluster of samples according to gene expression. Samples identified by patient number (n), specimen type (Bx, biopsy; RP, radical prostatectomy), and treatment status (Rx, treatment with imatinib mesylate).
regardless of processing protocol (Fig. 2C). All replicates, regardless of day of processing or overall chip quality, were adjacent to one another in the hierarchical cluster. Of the six patients with pretreatment and posttreatment microarray data, pretreatment and posttreatment specimens for four (patients 1, 6, 7, and 9) were located adjacent to each other, whereas the pretreatment and posttreatment samples from two (patients 3 and 10) were separated in the hierarchical cluster. This suggests that gene expression variation due to either differences in specimen handling (biopsies versus radical prostatectomy specimens) or imatinib mesylate is also less than the variation in gene expression between tumors for most specimens.

**Supervised analysis.** Supervised analysis can often identify significant gene expression associations between classes that are not obvious using unsupervised methods, such as hierarchical clustering or self-organized maps (20). Indeed, visual inspection of the normalized expression of the top 100 genes ranked according to their differential expression across treatment status suggested that imatinib mesylate was associated with differential gene expression (Fig. 3A). However, for individual genes, the degree of differential expression between the experimental classes (untreated versus treated) did not exceed that of randomly permuted class labels (at $P < 0.05$). As the small number of samples available for analysis compared with the large number of genes may limit the ability of any gene to outdo random permutation testing, we were concerned about having a false-negative finding and decided to use immunohistochemistry to validate differential expression for the highest ranked gene with increased expression following imatinib mesylate therapy.

**MKP1** (also called **DUSP1**), was the gene ranked highest using the signal-to-noise metric with a 6.1-fold difference in RNA expression on the microarrays (untreated, 320.66 ± 289.66; imatinib treated, 1,961 ± 1,321; Fig. 3B). Immunohistochemistry for MKP1 in prostate specimens using a previously validated antibody resulted in epithelial-specific staining (Fig. 3C). When pretreatment and posttreatment samples were compared for protein expression, MKP1 had increased expression in the treated compared with untreated prostate tumors ($P = 0.030$, two-tailed $t$ test; Fig. 3D). This suggests that microarray analysis following LCM and RNA amplification can detect differential gene expression, and permutation testing may be too stringent for data sets of limited size.

**Gene set enrichment analysis.** Gleaning mechanistic import from lists of differentially expressed genes identified by microarray analysis remains a significant challenge. We chose to apply GSEA to determine if the activity of specific cellular pathways were associated with imatinib mesylate therapy. When a collection of 540 pathway “gene sets” was tested using GSEA, only seven pathways had significant enrichment using a stringent false discovery rate of <0.25 (Fig. 4A). One, the “tsp1” pathway, entitled “Thrombospondin-1 induced apoptosis in microvascular endothelial cell,” had coordinately increased expression in the imatinib mesylate–treated samples with a low estimated false detection rate. Interestingly, when we review treated prostate cancer sections stained for CD31 and Apotag to look for evidence for endothelial cell apoptosis, there were areas within prostate cancer tumors staining positively for CD31 that also had ApoTag activity (Fig. 4B).

**Discussion**

Our results suggest that performing microarray analysis before and following neoadjuvant therapy is feasible and can help explore the biological effect of cancer therapies. To perform microarray analysis, the methods of LCM and RNA amplification had to be adapted so that sufficient material could be obtained from narrow-gauge biopsies. Here, we used samples collected from men with localized prostate cancer before and following treatment with imatinib mesylate and
show that technically introduced variability in expression is relatively small compared with the intertumor variability. Relative differences across tumors are also preserved as differential MKP1 expression was confirmed by immunohistochemistry. Finally, GSEA was applied to our sample set and provides a provocative, hypothesis-generating insight as to a potential tissue effect of imatinib mesylate. Our methods, similar to those used by an increasing number of investigators, seem sufficiently robust to minimize technically introduced gene expression artifact so that true differences in gene expression can be detected. Both control cell line RNA and replicate samples from tumors showed correlation that was significantly higher than the correlation between different tumors. Although the difference between tumors is likely due to a combination of biological variation and variation induced by differences in specimen handling, standardizing tissue collection procedures can help ensure that most observed variability in expression is biological.

Although we were unable to perform a more exhaustive validation using reverse transcription-PCR due to limited biopsy material, the successful validation of differential MKP1 protein expression provides compelling validation of the methods herein described. In addition, MKP1 expression in prostate cancer and prostatic intraepithelial neoplasia is inversely associated with apoptosis and up-regulation during imatinib mesylate therapy may represent an adaptive anti-apoptotic response (39, 40). Interestingly, GSEA identified the "mitogen-activated protein kinase cascade" and "tp1" as the top two pathways up-regulated in response to imatinib. These observations generate the plausible hypothesis that platelet-derived growth factor receptor-β blockade increases thrombospondin-1 expression in microvascular cells, which has been shown to cause cellular apoptosis through p38 mitogen-activated protein kinase activity (41). Although both endothelial and epithelial cells may respond to the thrombospondin-1 stimulation (and thus up-regulate the tsp1 pathway), the increased expression of MKP1 in epithelial cells may antagonize p38 mitogen-activated protein kinase-mediated apoptosis. This hypothesis unifies the changes in gene expression and immunohistochemistry induced by imatinib mesylate and is consistent with growing evidence that suggests imatinib mesylate’s action is likely through inhibiting platelet-derived growth factor receptor-β activity in pericyte and/or endothelial cells in tumor microvasculature (42–44).

Microarray analysis has been successfully applied to many basic and clinical questions and provided novel insights. Here, we adapted methods of LCM and RNA amplification to determine the feasibility of using microarray analysis to assess the effects of a targeted therapy. We found that the degree of technical variation introduced is much less than gene expression differences between tumors. In addition, even with a limited number of samples, expression analysis following LCM and RNA amplification identified MKP1 as a differentially expressed gene and thrombospondin-1-mediated apoptosis as a potential mechanism of action. These findings strongly support the feasibility and potential benefit of performing microarray analysis on minute quantities of tissue before and following targeted therapy.

References
Clinical Cancer Research

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