Expression signatures that correlated with Gleason score and relapse in prostate cancer

Marina Bibikova a, Eugene Chudin a, Amir Arsanjani b, Lixin Zhou a, Eliza Wickham Garcia a, Joshua Modder b, Monica Kostelec b, David Barker a, Tracy Downs b, Jian-Bing Fan a,⁎, Jessica Wang-Rodriguez c,⁎

a Illumina, Inc., 9885 Towne Centre Drive, San Diego, CA 92121, USA
b Division of Urology, Department of Surgery, and VA San Diego Healthcare System, USA
c Department of Pathology and VA San Diego Healthcare System, University of California at San Diego, 3350 La Jolla Village Drive, La Jolla, CA 92161, USA

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Abstract

Predicting prognosis in prostate carcinoma remains a challenge when using clinical and pathologic criteria only. We used an array-based DASL® assay to identify molecular signatures for predicting prostate cancer relapse in formalin-fixed, paraffin-embedded (FFPE) prostate cancers, through gene expression profiling of 512 prioritized genes. Of the 71 patients that we analyzed, all but 3 had no evidence of residual tumor (defined as negative surgical margins) following radical prostatectomy and no patient received adjuvant therapy following surgery. All of the 71 patients had an undetectable serum PSA following radical prostatectomy. Follow-up period was 44±15 months. Highly reproducible gene expression patterns were obtained with these samples (average $R^2 = 0.99$). We identified a panel of 11 genes that correlated positively and 5 genes that correlated negatively with Gleason grade. A gene expression score (GEX) was derived from the expression levels of the 16 genes. We assessed the prognostic value of these genes and found the GEX significantly correlated with disease relapse ($p=0.007$). These results suggest that the approach we used is effective for expression profiling in heterogeneous FFPE tissues for cancer diagnosis/prognosis biomarker discovery and validation.

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Progress in treating prostate cancer has been hampered by the finding that histologically identical cancers exhibit widely variant clinical behavior. The uncertainty regarding the appropriate clinical management of prostate cancer in many patients is related to an incomplete and unclear understanding of the molecular and genetic changes involved in prostate cancer development and disease progression. Since 1988, the routine use of serum PSA testing in men at risk for prostate cancer has led to more favorable disease characteristics at presentation (stage migration) [1] and earlier diagnosis and treatment [2]. Several investigators [3–5] have used these clinical parameters to stratify patients into risk groups (low, intermediate, high) and to predict clinical outcomes (nomograms). Despite these useful parameters, approximately 30% of patients with intermediate-risk prostate cancer fail standard treatment as evidenced by a rising serum PSA following definitive therapy. A better understanding of the molecular abnormalities that define these tumors at high risk for relapse is needed to help identify more precise biomarkers.

Comparison of gene expression patterns in different cell types, developmental stages, and disease states should enable the discovery of characteristic gene expression patterns that can be associated with functionally important states. Microarray-based tumor classification [6–9], as well as treatment response...
and clinical outcome prediction [10–12], has been demonstrated in many cancer types. Specifically, gene expression profiling has been used to assess a patient’s risk of failing therapy or to distinguish healthy prostate, benign prostatic hyperplasia, localized prostate cancer, and metastatic prostate cancer [10, 13–15]. These studies have demonstrated the feasibility of combining large-scale gene expression profiling with classic morphologic and clinical methods of staging and grading cancer for better diagnosis and outcome prediction [16–19].

Current microarray technologies typically require snap-frozen tissue. However, there is a vast supply of formalin-fixed, paraffin-embedded (FFPE) tissues for which clinical outcome is already known, and this makes them the most widely available sample source for prognosis studies [20–22]. The ability to analyze gene expression in archived tissues will greatly facilitate research in correlating gene expression profiles with given disease states, or histological and clinical phenotypes, and eventually in developing biomarkers for therapeutic decision making. This will also be useful for future clinical test development, as FFPE sample collection and storage is an easy and routine practice in most pathology laboratories.

In this study, we used an array-based DASL (cDNA-mediated annealing, selection, extension, and ligation) assay [50,51] to profile 512 candidate genes in 71 archived FFPE prostate carcinomas and identified a small set of genes whose expression patterns correlate highly with Gleason scores in the patients and are predictive of disease relapse. This approach has the potential to allow genomic analysis in patients with established clinical prostate cancer disease to predict disease outcomes.

Results

Gene expression profiling reproducibility

All the RNA samples assayed in this study were extracted from FFPE tissues. Two independent DASL assays were performed for each RNA sample; each used 200 ng total RNA [23]. To prequalify RNA samples prior to array analysis, we used a real-time PCR-based method to assess the intactness of the RNA samples. We find this approach more effective than using a combination of RNA quantitation and a gel-based size analysis. Specifically, 1 µl of the cDNA reactions was taken for a real-time PCR analysis of the housekeeping gene RPL13A. Highly reproducible gene expression profiles were obtained for the replicates of each FFPE sample ($R^2=0.99$), even though a wide range of RNA degradation was detected in these samples that had an up to 8-cycle difference in qPCR (i.e., ~170-fold difference in “PCR-able” RNA input) for the RPL13A gene (Fig. 1). We determined that samples should not exhibit a $C_t$ of more than 28 cycles under these conditions to ensure a reasonable expectation of reliable data in the DASL assay. In addition, similar expression profiles were obtained with 36 pairs of RNAs extracted independent of separate cuts of the same paraffin tissue blocks (average $R^2=0.93$) (data not shown). RT-PCR was performed to confirm some of the array results [24].

Correlation between gene expression signature and Gleason score

In the interest of having the tissue samples mimicking clinical situation as much as possible, we chose 71 tumors with various Gleason grades. Some tumors have uniformly one grade, while others have a primary and a secondary grade. The tumors with one grade were counted twice to comprise a Gleason score (i.e., Gleason grades 3+3= Gleason score 6). Gleason scores of two primary tumor patterns were the sum of the two Gleason grades (i.e., primary Gleason grade 4 and secondary Gleason grade 3 would have a Gleason score of 7). We used samples that contained at least 10% of the tumors as a cutoff for eligibility for DASL analysis.

We found differential gene expression of tumor samples with various Gleason grades, thus contributing to different clinical outcomes within the groups. We first identified genes that were either positively or negatively correlated with Gleason summary score (GS), using a permutation method (see Materials and methods for details), and generated a panel of 11 positively correlated genes, CCNE2, CDC6, FBP1, HOXC6, MKI67, MYBL2, PTTG1, DTL, UBE2C, WNT5A, and ALCAM, and 5 negatively correlated genes, AZGP1, CCK, MYLK, PPAP2B, and PROK1. Based on the expression profiles of these16 genes, a gene expression score (GEX; an expression analogy of Gleason grade) was calculated (see Materials and methods). The GEX had better correlation ($r=0.63$) with Gleason summary.

![Fig. 1. Sample QC (by qPCR) and array data quality assessment. $C_t$ was measured for a 90-bp amplicon from the RPL13A gene using SYBR green detection. $R^2$ represents the correlation between replicate DASL assays for the same cDNA run in parallel. Highly reproducible gene expression data are obtained with samples that have an up to 8-cycle difference in qPCR (i.e., ~170-fold difference in “PCR-able” RNA input) for a housekeeping gene, RPL13A. Samples are prequalified for array analysis by qPCR, using a $C_t$ number of 28 as the cutoff.](image-url)
score than did correlation of any individual gene (Fig. 2). The Pearson correlation coefficient between gene expression and Gleason score and the \( p \) value calculated from the permutation test are listed in Table 1. The 16 genes can be classified into several groups based on their biological functions: (1) proliferation, MKI67, MYBL2, WNT5A, PTTG1, AZGP1, and PROK1; (2) cell cycle, CCNE2 (cyclin E2), CDC6, MKI67, MYBL2, PTTG1, UBE2C; (3) differentiation, HOXC6, WNT5A; (4) cell adhesion, ALCAM, AZGP1, and MYLK; (5) signal transduction, WNT5A, CCK, MYLK, and UBE2C; (6) basic metabolism, FBP1, AZGP1, PPAP2B, and DTL (a protease).

To find out whether the GEX was significantly different in tumor versus nontumor samples, we profiled a total of 126 samples of FFPE cancer (\( N = 79 \)) and noncancer (\( N = 47 \)) prostate tissues. “Cancer” sections included 10–90% adenocarcinoma in the block. The mean GEX on the cancer tissues was 7.38 ±0.35 and the GEX on noncancer prostate tissues was 7.2 ±0.16 (\( p = 0.0013 \)), indicating that the GEX significantly correlated with the diagnostic tissues of cancer versus benign prostate tissues.

**Gene expression profiles to predict relapse of prostate cancer**

We assessed the prognostic value of the 16 GS-correlated genes. As shown in Fig. 3, there was a good correlation between the GEX and relapse and a near-linear increase in percentage of relapse cases with GEX between 7 and 7.6 (Fig. 3). For instance, when GEX was 7.4, approximately 75% of the cases relapsed. When the GEX reached 7.8, 100% of the cases relapsed. It is worth noting that the average GEX was 7.2 for GS7 patients without relapse and 7.4 for GS7 patients who relapsed, corresponding to 20 and 75% of the chance of relapse, respectively.

The receiver operating characteristic (ROC) curve showed that the 16-gene expression signature was more predictive of relapse than Gleason score (Fig. 4A). The GEX had an AUC (area under the curve) of 0.73, which was better than the Gleason score with an AUC of 0.65. Particularly, the GEX improved the relapse prediction in patients with a Gleason score of 7 (see Fig. 2).

Patients that experienced relapse tended to have higher GEX despite having identical Gleason scores. The most pronounced difference was observed in GS7 patients (two-sided \( t \) test \( p = 0.005 \), GS7-Y compared to GS7-N, Fig. 2). The GEX, when divided among the groups of GEX >7.3 and GEX ≤7.3 (cutoff was chosen as median GEX for GS7 and GS8 samples), had a significant correlation with subsequent

### Table 1

Sixteen genes that positively or negatively correlated with Gleason score and tumor content

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Correlation coefficient (Gleason score)</th>
<th>( p ) value (Gleason score)</th>
<th>Correlation coefficient (tumor content)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCNE2</td>
<td>0.3626</td>
<td>9.00 × 10^{-4}</td>
<td>0.1026</td>
</tr>
<tr>
<td>CDC6</td>
<td>0.3727</td>
<td>2.00 × 10^{-4}</td>
<td>0.3085</td>
</tr>
<tr>
<td>FBP1</td>
<td>0.3374</td>
<td>1.30 × 10^{-3}</td>
<td>0.5266</td>
</tr>
<tr>
<td>HOXC6</td>
<td>0.5038</td>
<td>0.00</td>
<td>0.7511</td>
</tr>
<tr>
<td>MKI67</td>
<td>0.3922</td>
<td>0.00</td>
<td>0.5582</td>
</tr>
<tr>
<td>MYBL2</td>
<td>0.3788</td>
<td>0.00</td>
<td>0.3844</td>
</tr>
<tr>
<td>PTTG1</td>
<td>0.3821</td>
<td>4.00 × 10^{-4}</td>
<td>0.2837</td>
</tr>
<tr>
<td>DTL</td>
<td>0.4485</td>
<td>0.00</td>
<td>0.2572</td>
</tr>
<tr>
<td>UBE2C</td>
<td>0.3251</td>
<td>1.10 × 10^{-3}</td>
<td>0.3127</td>
</tr>
<tr>
<td>WNT5A</td>
<td>0.3945</td>
<td>4.00 × 10^{-4}</td>
<td>0.1126</td>
</tr>
<tr>
<td>ALCAM</td>
<td>0.3512</td>
<td>1.40 × 10^{-3}</td>
<td>0.5657</td>
</tr>
<tr>
<td>AZGP1</td>
<td>−0.3549</td>
<td>2.00 × 10^{-4}</td>
<td>−0.2011</td>
</tr>
<tr>
<td>CCK</td>
<td>−0.3425</td>
<td>9.00 × 10^{-4}</td>
<td>−0.2737</td>
</tr>
<tr>
<td>MYLK</td>
<td>−0.3456</td>
<td>7.00 × 10^{-4}</td>
<td>−0.2950</td>
</tr>
<tr>
<td>PPAP2B</td>
<td>−0.3517</td>
<td>8.00 × 10^{-4}</td>
<td>−0.2800</td>
</tr>
<tr>
<td>PROK1</td>
<td>−0.3619</td>
<td>7.00 × 10^{-4}</td>
<td>−0.3048</td>
</tr>
</tbody>
</table>

![Fig. 2. Correlation between gene expression signature and Gleason score. 5-N, 6-N, 7-N, and 8-N correspond respectively to patient groups with Gleason score of 5–8 without relapse. 6-Y, 7-Y, 8-Y, and 9-Y correspond respectively to patient groups with Gleason score of 6–9 with relapse.](image1)

![Fig. 3. Plot of percentage of relapse cases vs the GEX.](image2)
Among the GS7 patients, 1/21 of GS 3+4 and 4/11 of GS 4+3 relapsed (Fisher exact test \( p = 0.037 \)). The mean GEXs were 7.236 and 7.305 for the two groups, respectively (\( p = 0.071 \) for hypothesis testing increased GEX for 4+3 patients). GS alone was associated with relapse versus no relapse (\( p = 0.02 \)). Neither the tumor stage nor the risk groups assigned at the time of biopsy significantly correlated with relapse (Kaplan–Meier analysis, \( p = 0.07 \) and 0.1, respectively).

We considered only samples (\( N = 71 \): 55 without relapse and 16 with relapse) with no residual tumor after surgery, with the exception of three cases that had nondetectable serum PSA after surgery but had positive margins, and excluded patients who received therapy and had no relapse. Therefore, the lack of relapse could not be due to selective therapies but the underlying biological difference among these patients.

GEX of the 34 matched, nontumor prostate tissues generated a mean of 7.19±0.18, lower than that in their tumor counterparts (7.25±0.25). However, the GEX on nontumor tissue of patients appeared to correlate with relapse by Kaplan–Meier analysis (\( p = 0.04 \)), although not as significantly as the tumor itself.

**Discussion**

In the past few years, numerous genome-wide gene expression profiling studies have been conducted in prostate cancers [8,10,13–15,25–38]. Specific gene-expression signatures have been identified for prostate cancer subclasses [26,30,32,38], cancer stages and Gleason score [35,37], and clinical outcomes [13–15,27,28,31,35]. In addition, the gene expression patterns further divided the prostate cancer into subgroups that correlated with the degree of tumor differentiation as well as patient survival [25,29,35]. However, it is no surprise that there have been significant differences among the molecular signatures identified in these studies, due to different sample sets (and sample quality), different array platforms or content, or different data analysis processes.

Biomarkers derived from multiple analyses on multiple microarray datasets clearly do not provide the level of confirmation necessary to translate into clinical utility. Using different methods on different samples is considered an excellent way to provide validation and to translate into broader clinical impact [9,17,18,39–41].

In this study, we took a candidate gene approach to identify genes that strongly correlated with Gleason score and the state of tumor differentiation and then used the expression signature to predict disease relapse. The candidate genes we profiled were largely selected from publicly reported lists of genes differentially expressed in prostate cancer. Therefore, our work in a way served as an independent validation for some of the previous studies. We selected FFPE tissue samples for study because of the abundant availability of archived material in a pathology laboratory with known long-term clinical outcome information. This study demonstrated that the DASL assay can overcome the challenge of analyzing small amounts of RNA isolated from clinical samples. The expression profiles generated appeared to be highly reproducible. The throughput and automated nature of the process made it a potential methodology for future clinical use.

In this study, we first identified 16 genes that strongly correlated with Gleason grade from samples of wide ranges of tumor enrichment (10–90%). One would argue that the tumor-specific expression signal should vary with the tumor content (among these samples). In fact, the expression level of 1 of the 16 genes (HOXC6) had a strong correlation with the tumor content in the samples (Table 1). However, there may be multiple factors that contribute to the Gleason grade of the tumor at the molecular level, such as stroma–tumor interactions. We believe the expression profiles of the 16 genes reflect contributions from both tumor cells and their environments; it is quite possible that nontumor cells may also contribute to the Gleason grade (please see below for more discussion). Moreover, combining the 16 genes together provides a balanced measurement of many different molecular contributions related to Gleason grade, including the tumor content.
The GEX derived from the expression levels of the 16 genes was used to predict relapse of prostate cancer. It is worth pointing out that there was no “training/fitting” made toward their prognostic power at the gene selection step. Making continuous analogy of Gleason grade increased molecular resolution, especially at GS=7–8, with which we can stratify patients better based on their gene expression profiles (Fig. 2); in turn, this translates to a good predictor of relapse for prostate cancer (Fig. 4).

Interestingly, the GEX exhibited a nonlinear pattern, in which the expression signature score stayed flat when GS < 7 and started rising at GS = 7 and plateaued at GS = 9 (Fig. 2). This suggests that there may be three distinct molecular stages among the prostate cancer patients, and this may have corresponded to Gleason scores 6, 7, and 8, respectively. GEX profiles can potentially identify a subset of histologically intermediate-grade tumors that have more aggressive clinical behavior, i.e., to separate out GS7 patients who were more likely to relapse.

The 16-gene signature may provide insight into the underlying molecular mechanisms that regulate the tumorigenesis and metastases in prostate cancer. For example, MKI67 is a known proliferation marker for prostate cancer [42] and other cancer types [43,44]. Activation of the Wnt signaling cascade associated with increased expression of WNT5A was suggested as a signature pathway of the early stage poor-prognosis subtype of human prostate cancer [28]. HOXC6 was identified previously as a good marker for prediction of prostate cancer patient outcome [35]. In our study, we found HOXC6 highly correlated with tumor content (Table 1). UBE2C is a cell cycle gene that promotes cell growth and malignant transformation. ALCAM plays an important role in progression of prostate cancer [45].

We also calculated the GEX in the “matched” nontumor prostate tissues in the same population. The GEXs were lower than those seen in the tumor glands but the scores had statistically significant correlation with disease relapse \( (p=0.04) \). However, the statistical significance was much less than the GEX in the tumor glands \( (p=0.007) \). It is possible that the most robust signals generated by GEX came from the tumor glands. However, it is highly plausible that the stromal signals also contributed to the overall GEX. Additional prospective clinical trials are needed to test whether nontumor stroma would generate signals that can predict relapse, i.e., the possibility of using small samples such as using LCM in needle biopsies, with or without tumor glands, to assess prognostic outcomes prior to definitive therapy.

The sensitivity and specificity of the 16 gene markers remain to be validated in another independent cohort and prospectively in clinical trials in which patients undergo prostate needle biopsy for diagnosis of carcinoma. We anticipate that this test would give us the information on diagnosis as well as prognosis through needle biopsy samples. The ultimate goal of this study is to develop a mature diagnostic test that is technically simple and applicable for routine clinical use and eventually incorporate it into existing prostate cancer nomograms [40,46,47].

### Materials and methods

#### Tissue selection and RNA extraction

Surgically removed radical prostatectomy specimens were processed under routine pathological protocol and examined by at least two pathologists. We obtained approval of UCSD IRB (No. 040487X) to study patients’ existing tissue materials and review of pertinent medical records. A study number was assigned to the specimen and the patient identification information (names and hospital identification number) was also recorded at the time of specimen retrieval. The specimens were received in the pathology laboratory fresh within 45 min of removal and fixed in 10% buffered formalin overnight. Representative sections were submitted for tissue processing and paraffin embedding. Five-micrometer-thick sections were made for routine hematoxylin and eosin stains. Specific tissue blocks that included areas of carcinoma and nearby noncancerous tissue were selected for RNA extraction. Tumor blocks from each case were chosen if they contained the predominant tumor grade and the most tumor volume. Each tissue block was reviewed by one of the authors, who is a board certified pathologist (J.W.R.), and co-authors (J.M., M.K., and A.A.) who were urology residents. Gleason grade and score assigned to each tissue block containing the tumor, and the percentage of tumor content was estimated and used as a reference for gene expression analysis. RNA was extracted from four or five 5-μm sections using an RNA extraction kit (Roche High Pure RNA Paraffin kit), yielding 0.5–3 μg of total RNA. RNA quality was assessed by quantitative RT-PCR analysis of the housekeeping gene RPL13A (forward primer, GTACGCTGTGAA GCCCATCAA, and reverse primer, GTTGGTGTTCATCCGCTTG).

Only tissue samples that yielded sufficient quantities and qualities of RNA were entered into the study. Samples were excluded if (1) the RNA was too degraded for study, (2) the block was less than 10% tumor, (3) the patient had a positive surgical resection margin and the patient’s postoperative PSA never nadired to below detectable level, or (4) the patient was treated with other adjuvant therapy. From the year 1998 to 2000, 71 of 150 cancer samples met above selection criteria and were entered into this study (Table 2). Three cases with positive margins were included in this cohort because their postoperative PSA nadired to undetectable levels; they were believed to be tumor free after the definitive resection. The patients were stratified into three clinical risk groups. Low-risk group patients \((n=29) \) had a serum PSA ≤ 10 ng/ml, Gleason summary score ≤ 6, and a digital rectal examination (DRE) of cT1c/cT2a.

#### Table 2

<table>
<thead>
<tr>
<th>Patient demographics</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years)</td>
<td>68.9 (55–81)</td>
</tr>
<tr>
<td>Mean PSA (ng/ml)</td>
<td>8.1 (1.76–24.03)</td>
</tr>
<tr>
<td>Months follow-up</td>
<td>43.9±15 (6–84)</td>
</tr>
<tr>
<td>Biopsy risk group ((n))</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>29</td>
</tr>
<tr>
<td>Intermediate</td>
<td>26</td>
</tr>
<tr>
<td>High</td>
<td>16</td>
</tr>
<tr>
<td>Gleason Score Distribution</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>7</td>
<td>31</td>
</tr>
<tr>
<td>8</td>
<td>26</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Relapse</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>55</td>
</tr>
<tr>
<td>Yes</td>
<td>16</td>
</tr>
<tr>
<td>Survival</td>
<td></td>
</tr>
<tr>
<td>Alive</td>
<td>60</td>
</tr>
<tr>
<td>Dead</td>
<td>11</td>
</tr>
<tr>
<td>AJCC TNM stage</td>
<td></td>
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<tr>
<td>I</td>
<td>0</td>
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<td>II</td>
<td>53</td>
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<tr>
<td>III</td>
<td>15</td>
</tr>
<tr>
<td>IV</td>
<td>3</td>
</tr>
</tbody>
</table>
Intermediate-risk group patients (n=26) had a serum PSA 10–20 ng/ml, Gleason summary score of 7, and DRE of cT2b/cT2c. The high-risk group patients (n=16) had a serum PSA >20 ng/ml, Gleason summary score of 8–10, and DRE of cT3a/cT3b. All tumor blocks contained at least 10% malignant glands. In addition, 34 matched, adjacent nontumor prostate tissues from the same cohort were used as the patients’ own baseline controls. These tissues were composed of inflammation, stroma, benign glandular hyperplasia, and glandular atrophy. As truly benign prostate controls, an additional 13 noncancer prostate tissues were used. They were from patients with benign prostate hyperplasia.

We had 6–84 months follow-up on the study patients. Information relevant to the patient’s diagnosis was obtained (Table 2), which included, but was not limited to, age, ethnicity, serum PSA at the time of surgery, tumor localization, pertinent past medical history related to comorbidity, other oncological history, family history of cancer, physical exam findings, radiological findings, biopsy date, biopsy result, type of operation performed (radical retropubic or radical perineal prostatectomy), TNM staging, neoadjuvant therapy (i.e., chemotherapy, hormones), adjuvant or salvage radiotherapy, hormonal therapy for a rising PSA (biochemical disease relapse), local vs distant disease recurrence, and survival outcome.

PSA recurrence was defined as two consecutive increasing PSA values of more than 1.0 ng/ml and differing by more than 0.2 ng/ml to avoid scoring PSA failure from PSA increase due to testosterone rebound, which can occur after the AST is discontinued. Before PSA recurrence, patients had a serum PSA measurement and an annual digital rectal examination at a median of every 4–6 months. After PSA recurrence, the serum PSA level was measured at a median of 3 months (range, 1 to 6 months).

Gene selection

In this study, we took a candidate gene approach. Instead of performing whole genome expression profiling, we focused on a set of “informative” genes that are the most relevant to prostate cancer (and other cancer types). The genes were selected based on two criteria: (1) Biological relevance. This included tumor suppressor genes and oncogenes; genes that were indirectly involved in cancer development, for example, DNA repair genes; metastasis-inhibitor genes; genes regulated by various signaling pathways and/or responsible for altered cell growth and differentiation or apoptosis. (2) Publicly reported lists of genes differentially expressed in prostate cancer [8,10,11,25–33,35,37,38,48]. We selected a list of 512 genes from these gene lists based on their overlapping occurrences among the studies, differential expression levels, and biological relevance.

High-throughput RNA profiling on universal array matrices

We used a gene expression profiling assay, the DASL assay, for parallel analysis of 1536 sequence targets (512 genes at three probes per gene) [23]. In this assay, two oligos were designed to target a specific gene sequence. Total RNA was first converted to cDNA by random priming. The corresponding query oligos annealed to the cDNA and were extended and ligated enzymatically. The ligated products were then amplified and fluorescently labeled using TaqMan chemistry and finally detected by binding to address sequences on the microarray. The ligated products were then amplified and fluorescently labeled using TaqMan chemistry and finally detected by binding to address sequences on the microarray.

Array data analysis

Array data were normalized using the “rank invariant” method in Illumina’s BeadStudio software. The method normalizes all arrays with respect to a common reference sample. For each array, normalization transforms array signals based on linear coefficients of robust least-squares fit (iteratively reweighted least squares using Tukey’s biweight functions) of intensities of a rank invariant set of probes. Specifically, all probes ranked between Low-Rank—90th and High-Rank—90th percentiles are considered. If the change in rank relative to the common reference is less than 0.05, the probe is considered to be rank invariant. If less than 2% of all probes are picked as rank invariant, LowRank is gradually decreased until it reaches the 25th percentile.

Samples with tumor content higher than 10% and inflammation content less than 5% were used to identify genes whose expression is correlated to Gleason grade. For each gene, we computed Pearson’s correlation coefficient between its expression level and its Gleason score. We assigned p values to observed correlations by a permutation test. Sample labels were randomly permuted 10,000 times and the correlation values were determined. For each gene, the p value was assigned as the fraction of random permutations that resulted in higher correlation value than the one seen with correct sample labels. We used a cutoff value of 14/10,000, which corresponded to a false discovery rate [49] adjusted p value of 0.05 and obtained a list of 16 genes. For all the selected genes, we fitted linear models (using the rlm function with method “MM” in the MASS library of the R statistical package) to predict Gleason grades and used the average of 16 independently derived predicted values, GEX, as a gene expression analogy of the Gleason grade. Kaplan–Meier analysis was performed using the SurvDiff function from the SURVIVAL library of the R package with parameters corresponding to a log-rank test.

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References


