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Novel Gene Expression Signature Predictive of Clinical Recurrence After Radical Prostatectomy in Early Stage Prostate Cancer Patients

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Abstract

BACKGROUND.—Current clinical tools have limited accuracy in differentiating patients with localized prostate cancer who are at risk of recurrence from patients with indolent disease. We aimed to identify a gene expression signature that jointly with clinical variables could improve upon the prediction of clinical recurrence after RP for patients with stage T2 PCa.

METHODS.—The study population includes consented patients who underwent a radical retropubic prostatectomy (RP) and bilateral pelvic lymph node dissection at the University of Southern California in the PSA-era (1988–2008). We used a nested case-control study of 187 organ-confined patients (pT2N0M0): 154 with no recurrence (“controls”) and 33 with clinical recurrence (“cases”). RNA was obtained from laser capture microdissected malignant glands representative of the overall Gleason score of each patient. Whole genome gene expression profiles (29,000 transcripts) were obtained using the Whole Genome DASL HT platform (Illumina, Inc). A gene expression signature of PCa clinical recurrence was identified using

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stability selection with elastic net regularized logistic regression. Three existing datasets generated with the Affymetrix Human Exon 1.0ST array were used for validation: Mayo Clinic (MC, n = 545), Memorial Sloan Kettering Cancer Center (SKCC, n = 150), and Erasmus Medical Center (EMC, n = 48). The areas under the ROC curve (AUCs) were obtained using repeated fivefold cross-validation.

RESULTS.—A 28-gene expression signature was identified that jointly with key clinical variables (age, Gleason score, pre-operative PSA level, and operation year) was predictive of clinical recurrence (AUC of clinical variables only was 0.67, AUC of clinical variables, and 28-gene signature was 0.99). The AUC of this gene signature fitted in each of the external datasets jointly with clinical variables was 0.75 (0.72–0.77) (MC), 0.90 (0.86–0.94) (MSKCC), and 0.82 (0.74–0.91) (EMC), whereas the AUC for clinical variables only in each dataset was 0.72 (0.70–0.74), 0.86 (0.82–0.91), and 0.76 (0.67–0.85), respectively.

CONCLUSIONS.—We report a novel gene-expression based classifier identified using agnostic approaches from whole genome expression profiles that can improve upon the accuracy of clinical indicators to stratify early stage localized patients at risk of clinical recurrence after RP.

Keywords

gene expression; stage II; clinical recurrence; stability selection

INTRODUCTION

In the United States, it is estimated that one in six men will be diagnosed with prostate cancer (PCa) in their lifetime and approximately 80% are diagnosed with tumors confined to the prostate. Most localized PCa tumors are indolent and will never become aggressive during a patient's lifetime. However, to date, over half of localized PCa patients have undergone radical prostatectomy (RP) as their primary treatment choice [1]. Despite treatment, approximately 20% of these patients may continue to experience a rising PSA level after surgery (biochemical recurrence) of which 20–30% will develop metastasis and PCa-related death [2,3]. Therefore, distinguishing men diagnosed with localized disease but still at risk of progression from men with localized PCa who will not progress is a pressing priority in the clinical treatment of PCa. This would allow for early identification of men with truly indolent tumors who can avoid RP and enroll in active surveillance and those with aggressive tumors who will benefit more from definitive treatment and/or more aggressive and earlier interventions.

Current prognostic tools to determine risk of progression use clinical variables, such as Gleason score, stage, pre-operative PSA level, but these variables have limited predictive accuracy [4–7]. Tumor biomarkers used jointly with existing clinical variables have been shown to provide additional information to accurately differentiate aggressive and indolent primary PCa tumors [8–11]. Previous studies indicate that within tumors histologically classified as non-aggressive, subsets of cells present at the time of diagnosis may harbor gene expression profiles characteristic of cells with metastatic potential that can be predictive of clinical recurrence [12–20]; however, few of these profiles have been adopted in the clinic for further validation [13,14,17]. Moreover, few of these studies utilized whole

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genome gene expression data [14,20] and/or utilized tissue microdissection to account for tumor heterogeneity in RNA sampling [12,18,20]. Therefore, there is still a need for novel tumor biomarkers that can help improve prediction of prostate cancer recurrence upon clinical variables.

We report a novel gene expression-based profile that improves the prediction of PCa clinical recurrence over clinical variables alone. This predictive signature was identified using whole genome expression data (over 27,000 coding transcripts and over 1,500 non-coding transcripts) obtained from microdissected malignant prostate glands from 187 prostate cancer patients diagnosed with organ-confined disease (stage T2a-T2c), treated with radical prostatectomy at the USC Norris Comprehensive Cancer Center, Department of Urology, who either developed clinical recurrence, or remained disease free after a comparable follow-up time.

MATERIALS AND METHODS

Patient Population

This study included patients diagnosed with organ-confined disease (pT2) who underwent a radical retropubic prostatectomy and bilateral pelvic lymph node dissection (RRP/PLND) at the University of Southern California from 1988 to 2008 (within the PSA-era at the institution) (n = 2,646). After surgery, patients who consented to enroll in this patient database were followed every 4–6 months in year 1, every 6 months in years 2 and 3, and once annually afterward. During the visits, patients received a physical examination, had a serum PSA measurement, and chest x-ray. Bone scans were also completed if there were signs of progression, such as an increase in PSA levels. Biochemical recurrence (BCR) was defined as a detectable PSA level based on the era-specific assay's detectability limit, verified by two consecutive increased PSA tests, with 3–4 months in-between blood draws [21]. Patients were defined as having clinical recurrence (CR) after the detection of recurrent local or distant disease by imaging. All specimens from radical prostatectomies were assessed using consistent pathological reporting.

Patients from this cohort were selected for this nested case control study. Eligible participants did not have lymph node involvement (N0), but had formalin-fixed paraffin-embedded (FFPE) prostatectomy tissue available for processing, and also had available clinical and follow-up data. Among the 2,552 eligible patients, there were 2,359 patients who had no evidence of disease after surgery (NED), 147 with biochemical/PSA recurrence only (BCR), and 46 with clinical recurrence (CR). Since some BCR patients do not ever experience metastatic disease, in order to enrich for genes predictive of truly aggressive PCa in this study, we compared NED and CR patients to determine a gene expression signature predictive of aggressive disease. Our final sample set included 154 NED patients and 33 with CR (those with tissue blocks available for microdissection).

Laser Capture Microdissection and RNA Extraction

All FFPE prostatectomy tissue sections were reviewed by an expert pathologist (Dr. Andy Sherrod) with the primary goal of determining the densest region of tumor to capture as

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much tumor RNA as possible, which usually translates into the region of higher Gleason score/grade of the tumor. In order to enrich for malignant glands and avoid contamination with stromal tissue or non-malignant glands, a laser capture microdissection (LCM) microscope (Arcturus® Laser Capture Microdissection, Model Veritas; Applied Biosystems by Life Technologies, Foster City, CA) was used to microdissect malignant prostate glands. Tumor sections cut at 5 microns were lightly stained with hematoxylin and eosin prior to microdissection. RNA extraction was performed using the Qiagen AllPrep DNA/RNA FFPE kit (Qiagen, Valencia, CA).

Gene Expression Microarray

Genome-wide gene expression profiles were generated for all samples, 50–200 ng RNA each, using the Whole-Genome DASL-HT Assay (Illumina, Inc.) [22]. The HumanHT-12 v4 BeadChip was used to detect the following transcripts using the RNA from the tumor samples: 27,253 coding transcripts (well-established annotations), 426 coding transcripts (provisional annotations), 1,580 non-coding transcripts (well-established annotations), and 26 non-coding transcripts (provisional annotations) (Illumina; Whole-Genome DASL ® HT Assay for Expression Profiling in FFPE Samples; Data Sheet: RNA Analysis, 2010). For quality control purposes, 20% of samples were included as duplicates and an even number of cases and controls were run on the same array chip. Technical replicates that were used to measure the variation induced by the processing of samples showed very good reproducibility ($r^2 = 0.97$). Three of the samples processed had low sensitivities (~2,000–4,000 genes detected) based on the *P*-value thresholds and therefore were not included in further analyses.

Pre-Processing of Gene Expression Data

All pre-processing of data and subsequent analyses were performed using R and Bioconductor [23]. Control probes and sample probes were used to preprocess (normalization and background correction) and to assess quality control using Bioconductor's *lumi* and *limma* packages. A specific pre-processing package (*neqc*) allowed for non-parametric background correction followed by quantile normalization using both control and sample probes [24]. This method provides the optimal compromise between precision and bias that occurs when using algorithms in preprocessing. We further considered and adjusted for possible batch effects by chip array during the microarray processing using ComBat [25]. The adjustment also took into account any batch effects by shipment since each shipment of RNA samples sent to Illumina involved the use of several BeadChips.

Differential Gene Expression

We identified differentially expressed genes (DEGs) between tumors of NED patients (n = 154) and CR patients (n = 33) using the empirical Bayes moderated *t*-test [26], which was applied on the entire set of ~29,000 features, adjusting for age (coded as a continuous variable), pre-operative PSA level (continuous), pathologic Gleason score (≤6, 7, 8–10), neoadjuvant hormone therapy (no, yes), operation year (continuous), and surgical margin status (positive, negative). Multiple testing correction was done by calculating the False Discovery Rate (FDR) using the Benjamini–Hochberg method [27].

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Pathway Analyses

Using the resulting DEGs obtained from the analysis, GeneOntology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed using WebGestalt software (WEB-based GEne SeT AnaLysis Toolkit) (Nashville, TN). [28–30] Pathway analyses were completed for all differentially expressed genes (DEGs) and separately for genes that had higher expression in tumors of CR patients compared to NED patients (hereafter, referred to as up-regulated genes) and for genes that had lower expression in tumors of CR patients compared to NED patients (hereafter, referred to as down-regulated genes). All WebGestalt gene enrichment analyses were done using the following parameters: Benjamini–Hochberg correction for multiple testing, minimum of four genes within each category, and a significance level of multiple tested corrected $P < 0.05$. Ingenuity Pathway Analysis (IPA) was also used to gain further insight into the gene networks, canonical pathways, diseases, and functions associated with the genes, and upstream regulators using the DEGs.

Identification of Gene Signatures Predictive of Aggressive PCa

Using the pre-processed list of 29,000 targets, we identified a multivariate risk prediction model for PCa clinical recurrence using stability selection with elastic net-regularized logistic regression [31]. We used the R Bioconductor package *caret* to calibrate the optimal tuning parameter, using elastic net with repeated 10-fold cross-validation, and settled on an $\alpha = 0.2$ as this would maximize the AUC (area under the curve) estimate while allowing for inclusion of as many possible features while maintaining good prediction. Using this tuning parameter, we implemented stability selection using 500 subsamples using the package *glmnet* in R, with each subsample having half of the sample size (original sample size n = 187). We used different frequency thresholds from 20 to 80% to determine the most predictive features. The different models identified were evaluated by estimating the average AUC across repeated (10 times) fivefold cross-validation. The following clinical variables were force-included in all models: Gleason, PSA, year of operation, and age at surgery. In addition, we also included use of neo-adjuvant androgen deprivation therapy (NADT), as a possible variable for selection, although not forced-included. A flowchart of the statistical analyses undertaken is shown in Figure 1.

In Silico Validation of Gene Signature

For validation of the identified models, we used three external datasets from three different studies that used whole-genome gene expression of PCa tumors. These datasets were: (i) from the Mayo Clinic (MC) [14]; (ii) Memorial Sloan-Kettering Cancer Center (MSKCC) [32]; and (iii) Erasmus Medical Center (EMC) [33]. Genomic and clinical data for these studies were obtained through the National Center of Biotechnology Information (NCBI) database repository for genomic data, Gene Expression Omnibus (GEO) (series accession numbers: GSE46691, GSE21032, and GSE41410). All three studies used the Affymetrix Human Exon 1.0 ST array to obtain gene expression data. This array consists of ~1.4 million probe sets, with approximately four probes per exon and about 40 probes per gene. In order to perform validation using these datasets, all probes and expression profiles corresponding to the genes in our predictive gene signature were extracted. Partek® Genomics Suite

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(Partek Inc., St. Louis, MO), was used to extract the raw data (Affymetrix CEL files) from GEO and was normalized through standard robust multi-array average (RMA) method and background correction for Affymetrix arrays. In order to ensure that all possible probes with good reliability were included in the validation, extended and full annotations were obtained for all probes pertaining to genes in the model. Probes from the full probeset annotation were used for final validation, since probe intensity distributions among extended and full probes are almost indistinguishable [34]. Using corresponding expression data from the patient population from each of the studies, repeated fivefold cross-validation using elastic net ($\alpha = 0.2$ and no standardization of the probe variables) was performed for validation. To determine the best prediction of a parsimonious model, the average AUC across all cross-validation runs was obtained using the LASSO penalty parameter set as one standard error above the detected minimum penalty, which indicates the lowest cross-validation error. Genes for all the possible predictive models generated from the stability selection (frequency threshold 20–80%) were assessed using the probe and clinical data available for each dataset.

RESULTS

Whole-genome gene expression profiles were generated for 154 patients who had no evidence of disease (NED) following surgery after at least 2 years of follow-up, and 33 patients who experienced clinical recurrence of disease, with local or distal metastasis detected (CR). Compared to NED patients, CR patients had higher Gleason score (Gleason 8–10, 36% CR vs. 16% NEDs, $P = 0.01$), and more had neoadjuvant hormonal therapy prior to surgery (24% CR vs. 4% NEDs, $P = 0.001$) (Table I). CR patients were also more likely to be classified as high-risk according to the D'Amico risk classification using available diagnostic data prior to surgery. The median follow-up time was 9.55 years for NED patients and 5.83 years until clinical recurrence for CR patients. There were no differences in racial/ethnic distribution between the NED and CR patients, with the majority of patients being non-Hispanic White (89% and 88%, respectively).

Identification of Differentially Expressed Genes (DEGs)

There were 184 differentially expressed features/probes, which represent a total of 172 differentially expressed genes (DEGs), in comparisons of tumors from CR and NED patients (Supplementary Table SI). All but 10 genes had a fold change of >1.2 with FDR-corrected P-values < 0.05.

Gene Set Enrichment Analyses

We did enrichment analyses for GeneOntology (GO) categories for all DEGs between tumors of CR patients and NED patients and identified eight functional categories enriched for DEGs that related to bioenergetics, with the three terminal categories being: NADP binding (four genes), oxidoreductase activity acting on the CH-OH group of donors with NAD or NADP as acceptor (six genes), and growth factor binding (five genes) (Supplementary Fig. S1a, Table SII). When considering DEGs with lower expression in tumors of CR patients compared to NED patients, we identified a similar pattern with seven enriched GO categories, with the three terminal categories being: coenzyme binding

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(six genes), oxidoreductase activity acting on CH-OH group of donors, NAD or NADP as acceptor (five genes), peptide binding (five genes) (Supplementary Fig. S1b, Table SII). There were two enriched GO categories for DEGs with higher expression in tumors of CR patients compared to tumors of NED patients, and the one terminal category included five genes in growth factor binding (Supplementary Fig. S1c). Further analyses using *KEGG* annotation identified three pathways enriched among all DEGs: metabolic pathways (12 DEGs), regulation of actin cytoskeleton (four DEGs), and pathways in cancer (four DEGs) (Supplementary Table SIII).

Disease-enrichment analyses showed enrichment of DEGs annotated to 27 disease categories, with the highest enrichment, based on the ratio of observed number of genes over the expected (R), found in the “fractures, bones” category (four genes). Among these categories, nine were directly related to cancer, including “prostatic neoplasms” (four DEGs: REPS2, CTBP2, LDAH, ANO7) (Supplementary Table SIV). We summarize in Table II all 33 DEGs identified as part of the terminal enriched pathways through GO and KEGG annotations, and genes found to be enriched as part of “prostatic neoplasms.” Among these 33 DEGs, eight were previously reported to be involved in PCa tumorigenesis (*ADI1*, *ANO7*, *BMPR2*, *CTBP2*, *DCXR*, *LDAH*, *NRP1*, *REPS2*) and 17 were reported to be involved in tumorigenesis of other cancers.

In order to identify candidate chromosomal deletions or amplifications directly responsible for differential gene expression, we analyzed whether there were specific genomic locations enriched for DEGs, as this may identify chromosomal deletions or amplifications. When all DEGs were considered, four cytogenetic bands were identified: (i) Chr3q: 10 DEGs; (ii) Chr12q: 11 DEGs; (iii) Chr18q: 6 DEGs, and (iv) Chr7q22: 4 DEGs. No chromosomal regions were found to be enriched when considering only upregulated or down-regulated DEGs (Supplementary Table SV).

We next used enrichment analysis to identify candidate miRNAs that may regulate the expression of DEGs. For all DEGs, there were 27 significantly enriched miRNA. Among them, we identified MIR-506 as a putative regulator of 13 DEGs and MIR-181, as a putative regulator of 11 DEGs. All other identified DEGs were identified as putative regulators of 4–7 DEGs. For DEGs with higher expression in CR patients compared to NED patients, there were 12 enriched miRNAs, of which nine overlapped with the miRNA found for all DEGs. For DEGs with lower expression in CR patients compared to NED patients, there were four enriched miRNAs that overlapped with the miRNA found for all DEGs (Supplementary Table VI).

Additional analysis included exploring transcription factor (TF) binding sites associated with the regulation of the genes in the lists of DEGs. For the list of all DEGs, there were 117 significantly enriched TF binding sites, 32 for DEGs with higher regulation among tumors of CR patients compared to tumors of NED patients, and 36 for DEGs with lower expression in the same group comparison (Supplementary Table SVII)

Finally, we investigated the associated small molecule targets given the set of DEGs. For all DEGs, seven categories were found, which adenine being the one with most targets

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[7], followed by NADH [6], and adenosine [5]. For DEGs with higher expression in tumors of CR patients compared to tumors of NED patients, we identified only one small molecule (glycine), and four for DEGs with lower expression in the same group comparison (Supplementary Table SVIII).

Gene Network Analyses

We used IPA software (IngenuitySystems) to analyze functional relationships among DEGs. Each generated network includes a score based on the negative log of the P-value calculated from the Fisher's Exact t-test used to indicate the likelihood of the network generated by random chance alone. The three IPA networks identified for all DEGs with a score >40 include: (i) embryonic development, organismal survival, cell death, and survival; (ii) organismal development, cancer, organismal injury, and abnormalities; (iii) cell morphology, cellular development, cellular growth, and proliferation. For each of these networks, we noted the following main "regulatory hubs" (genes predicted to regulate multiple DEGs): NF-kb (network 1), ERK and ERK1 (network 2), Akt (network 3) (Supplemental Fig. S2). The one network identified for DEGs with lower expression in tumors among CR patients versus tumors of NED patients with a score >40 was cellular assembly and organization, cellular compromise, cellular movement. The three networks identified for DEGs with higher expression in the same comparison include: (i) cancer, organismal injury, and abnormalities, reproductive system disease; (ii) molecular transport, nucleic acid metabolism, small molecular biochemistry; (iii) digestive system development and function, organ morphology, organismal development (Supplemental Fig. S2).

Gene Expression Signature Predictive of Aggressive PCa

Using stability selection with elastic net regression at different thresholds (20–80%), we identified eight different models. The model with 28 genes identified at 50% frequency threshold showed the highest AUC after repeated cross-validation and therefore was selected as the most predictive model (Table III). AUCs for the genes obtained at thresholds of 60–80% while including all clinical variables ranged from 0.80 to 0.92. After repeated cross-validation, a model with only clinical variables (Gleason score, PSA, operation year, and age at diagnosis) predicted CR with an AUC of 0.60 whereas the same model with the addition of the 28 genes increased the AUC to 0.97. A heat map of the 28 gene signature contrasting cases and control is shown in Supplemental Figure S3.

In Silico Validation of the 28-Gene Signature

To further assess the predictive ability of the 28-gene signature, we identified three external datasets with whole genome data that included appropriate outcomes (MC, MSKCC, and EMC), which we used for in silico validation. Across all datasets, the 28-gene signature improved upon the predictive ability (AUC) of the available clinical variables alone. Specifically, using the MC dataset, the 28-gene model with Gleason score yielded an AUC = 0.75, a 3% increase above AUC = 0.72 in the model with only Gleason score (no other clinical variables were available). Using the MSKCC expression data, the 28-gene model with clinical variables without any missing data (age at diagnosis, race/ethnicity, neo-adjuvant, and adjuvant treatment status) obtained an AUC = 0.90, a 4% improvement over clinical variables alone with AUC = 0.86. With the EMC dataset, the 28-gene model

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with the available clinical variables (Gleason score and pathologic T stage) yielded an AUC = 0.82, a 6% improvement over clinical variables only with an AUC = 0.76 (Table IV).

DISCUSSION

In this study, we report the identification of a 28-gene expression-based signature that improves the predictive ability of clinical recurrence upon a model with clinical variables alone, in both the original dataset and in three independent datasets. This signature was identified using whole genome gene expression of 187 resected tumors from radical prostatectomy patients diagnosed with organ-confined prostate cancer (stage pT2N0M0) with extensive follow-up. Our dataset included 154 prostate tumors from patients with no evidence of disease (NED) after surgery and 33 tumors from patients who experienced clinical recurrence (CR) after surgery. The results from this study provide novel data that with further validation may contribute to more accurate assessment of prognosis and thus aid in appropriate treatment decisions among patients diagnosed with early stage organ-confined localized disease.

When comparing whole genome expression profiles of tumors of CR patients to NED patients, we identified 172 statistically significant differentially expressed genes (DEGs). Among these DEGs, we observed enrichment in several key pathways defined by molecular function, biological function, and/or disease association. These enriched pathways include various metabolic pathways, including methionine and glucose metabolism, several localizing to the mitochondria, cell proliferation, cell motility and migration, and membrane trafficking. Altogether, these enriched pathways included 33 of the 172 DEGs. Among these 33 DEGs, there were eight genes previously reported to be relevant for prostate carcinogenesis: *ANO7*, *ADI1*, *BMPR2*, *CTBP2*, *DCXR*, *LDAH*, *NPR1*, and *REPS2*. *ADI1* and *ANO7* are two androgen-responsive genes reported to play a suppressor role in PCA progression and tumor invasion [35–37], and to inversely correlate with Gleason grade inversely correlate with Gleason grade [38–40], respectively. *REPS2* has also been reported to be involved in PCa cell proliferation and to be down-regulated during PCa progression [41–43]. Consistent with these functions, we found reduced expression of *ADI1*, *ANO7*, and *REPS2* in CR tumors compared to NED tumors. *BMPR2*, which we found to be up-regulated in CR patients compared to NED, has been reported to play a role in PCa cell invasion [44]. *CTBP2* is reported to have two isoforms with different functions, was found to promote PCa cell proliferation and progression, and to also act as a transcriptional repressor [45–47]. Herein, we found this gene to have reduced expression in CR compared to NED tumors, which is in contrast to what has been reported previously for PCa [45]. *DCXR* has been reported to be a biomarker of PCa [48] in addition to playing a role in hepatocellular carcinoma [49] and melanocyte lesions [50]. We observed that CR tumors had lower levels of *DCXR* expression compared to NED. *NRP1*, which we also found to have higher expression in CR tumors, has been reported to participate in cell migration and survival and in predicting bladder cancer progression and prostate cancer relapse [51,52]. Finally, we observed lower expression of *LDAH*, a gene reported to associate with PCa risk [53,54].

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Based on our GO analysis of the DEGs, there was enrichment for three main molecular function categories: oxidoreductase activity, NADP binding, and growth factor binding with the first two categories being mostly driven by down-regulated genes, and the latter being mostly driven by up-regulated genes in CR compared to NED patients. Among DEGs identified as part of the oxidoreductase activity, two were previously discussed genes, *CTBP2* and *DCXR*, as well as four additional genes involved in several metabolic reactions, *CYB5A* (ferrous hemoglobin metabolism), *GRHPR* (pyruvate metabolism), *MAT2B* (S-adenosyl methionine biosynthesis), and *ME2* (malic acid metabolism). *CYP5A*, *GRHPR*, and *ME2* expression have been reported to be de-regulated in pancreatic cancer, liver cancer, and melanoma, respectively [55–57]. *MAT2B* participates in the recruitment of MEK and ERK during tumorigenesis in several cancers [58]; consistent with this function we observed overexpression of this gene in CR tumors compared to NED tumors.

Among genes enriched in the binding molecular function category were the previously discussed *GRHPR* as well as *DUOX1*, *DECR1*, and *FMO5*. *DUOX1* is a hydrogen peroxide producer and participates in cell migration and antimicrobial defense [59]. *DECR1* and *FMO5* are related to mitochondrial function, the former participates in fatty acid metabolism in the mitochondria and the second one was reported to associate with mitochondrial DNA (mtDNA) levels [44]. There were five genes enriched in the growth factor binding GO category: *COL1A2*, *ESM1*, *RHBDF2* and the two previously discussed *BMPR2* and *NRP1* genes. *COL1A2* encodes for a type I collagen chain reported to associate with proliferation and migration of breast cancer cells [60]; consistent with this role, we observed this gene had higher expression in CR compared to NED tumors. *ESM1* is an endothelial-specific factor reported to be a possible biomarker for gastric and breast cancer and associated with breast cancer invasiveness [61,62]. *RHBDF2* is a protease reported to associate with ovarian cancer progression [63].

KEGG pathway enrichment analyses identified metabolism, pathways in cancer, and regulation of actin and cytoskeleton as being enriched for DEGS. Among DEGs in the metabolic pathways, there were four genes previously discussed (*ADI1*, *CCXR*, *GRHPR*, *MAT2B*) and eight additional genes. Among them, two participate in metabolic reactions that take place in the mitochondria: *ACAFSB* (fatty acid metabolism), *GLDC* (glycine metabolism); and five others participate in various metabolic pathways: *AGPAT2* (phospholipid synthesis), *ALDH1A2* (retinoic acid synthesis), *CHPT1* (phosphatidylcholine metabolism), *NADSYN1* (NAD synthesis), and *SRM* (spermidine synthesis). Finally, we identified *SYNJ1*, which is involved in membrane trafficking. Three of these 12 genes have been previously reported to be associated with tumorigenesis of osteosarcoma (*AGPAT2*) [64], breast cancer (*CHPT1*) [65], and lung cancer (*GLDC*) [66]. Within this enriched KEGG category, we highlight the cysteine and methionine metabolism pathway, involving *MAT2B*, *SRM*, and *ADI1*, which have been shown to associate with cancer development [67,68].

Among genes identified as part of the KEGG Pathways in Cancer was *CTBP2*, previously discussed, *CDKN2B*, involved in cell proliferation and reported to associate with several cancers [66], *LAMA3*, which participates in base membrane formation and cell migration and was reported to associate with gastric cancer [66], and *PIAS4*, which participates in

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autophagy and was reported to associate with pancreas cancer [66]. Among genes enriched in the KEGG actin/cytoskeleton regulation pathway were two genes that participate in cell migration: *MYH9* and *TMSBX4* both previously reported to associate with head and neck cancer [69] and several cancer types [70–72], respectively. In addition, this pathway was enriched by one G-protein gene (*ARHGEF6*) and a gene that participates in cell mobility and angiogenesis (*BAIAP2*).

The networks generated from the pathway analysis of the DEGs identified several central genes that act as "hubs" in these pathways and have been reported to be involved in cancer development and progression. Among them were NF-κβ, VEGF, ERK1 (MAPK3), and Tgfβ, Akt (protein kinase B), PI3 K, Ras, and EGFR. NF-κβ, EGFR has been reported to be involved in several cancers, including prostate cancer where its increased signaling has been reported to be involved in stem-like human prostate tumor-initiating cells and progression of disease [73–75]. De-regulation of VEGF, TGFβ, EGFR, and PI3 K/Akt have been reported as important steps in cancer invasion and metastasis, including prostate cancer [76–78]. Alterations of the Ras oncogene and MAPK3 have been reported to play an important role in the progression of prostate cancer cells to androgen resistance [79,80]. Similarly, our miRNA enrichment analyses identified an enrichment of DEGs that are regulated by many miRNAs known to be associated with prostate cancer progression [81].

We validated the predictive performance of our 28-gene model in three separate datasets that included whole-genome expression profiles that were obtained using a different platform than the one used in this study. Using data from all three datasets, the 28-gene model improved upon the prediction of clinical recurrence over models that included clinical variables only, with improvements ranging from 3 to 6%. Of the three datasets, the most comparable to our study design was the one from the Mayo Clinic. The AUC obtained for the 28-gene signature including Gleason score, which was the only clinical variable publicly available in the Mayo Clinic dataset, (AUC = 0.75; 0.72–0.77), was identical to the AUC reported by investigators from the Mayo Clinic and GenomeDx, for their 22-gene signature (AUC = 0.75; 0.68–0.83). We observed a comparable improvement in AUC when comparing the model with only clinical variables to the model that included both clinical and genetic components [14]. A key difference between our model and the GenomeDx one is that ours was identified among purely organ-confined prostate cancer patients with stage T2, although we note that the majority were stage T2C. In contrast, this Mayo clinic dataset included patients with stages higher than stage II, had a dissimilar distribution of pathological stage between cases and controls, and included lymph node positive patients.

Our study has several strengths. One, the use of microdissected tissue to enrich for malignant glands representative of the tumor's Gleason grade, which ensured the obtainment of gene expression profiles not contaminated with non-malignant tissue. Second, we used tumors from a well-annotated cohort with extensive and active follow-up. Third, we focused our analyses to organ-confined cancer patients of stage pT2, which allowed for comparisons between cases and controls without biases introduced by differences in stage distribution, and the identification of gene profiles representative of very early PCa. Finally, we used rigorous statistical methods to minimize variability in order to capture the most predictive genes of metastatic disease. Among the limitations of this study is the

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possibility that tumor heterogeneity within the prostate may not have allowed for proper sampling of the foci most representative of the tumor's potential to progress. This fact would introduce misclassification in our sample, and given that this misclassification would be non-differential with respect to case or control status (as both are equally likely to show heterogeneity), it could bias our findings toward the null. Therefore, we are more likely to have missed important associations between expression profiles and clinical recurrence rather that reporting inflated associations. We also acknowledge the relatively modest number of metastatic cases (n = 33). In spite of the large size of our cohort, few stage II patients experience a clinical recurrence. We sought to address this by validating our model in a dataset with higher numbers of clinical metastatic patients and obtained promising results that show an improvement of prediction of our model compared to clinical variables only. Lastly, for the original discovery of the 28-gene signature, given the modest number of patients with recurrence, we used cross-validation to estimate the AUC. Given that we used the same dataset used for discovery, our estimated AUC is likely an overestimate due to overfitting. However, the external validation datasets confirm that a model with clinical variables and the 28-gene signature is more predictive than clinical variables alone. Therefore, additional validation studies will be needed to confirm our findings and to obtain more accurate estimates of predictive accuracy. Moreover, future validation studies are needed to determine the utility of this signature to predict adverse pathology and risk of cancer progression at the time of initial biopsy.

CONCLUSIONS

In summary, we report a 28-gene model that used in conjunction with clinical variables improves the prediction of clinical recurrence among early stage localized PCa. This model, once validated in additional external databases, may aid clinicians in identifying patients with early localized disease at high risk of recurrence who may benefit from more aggressive treatments at the time of radical prostatectomy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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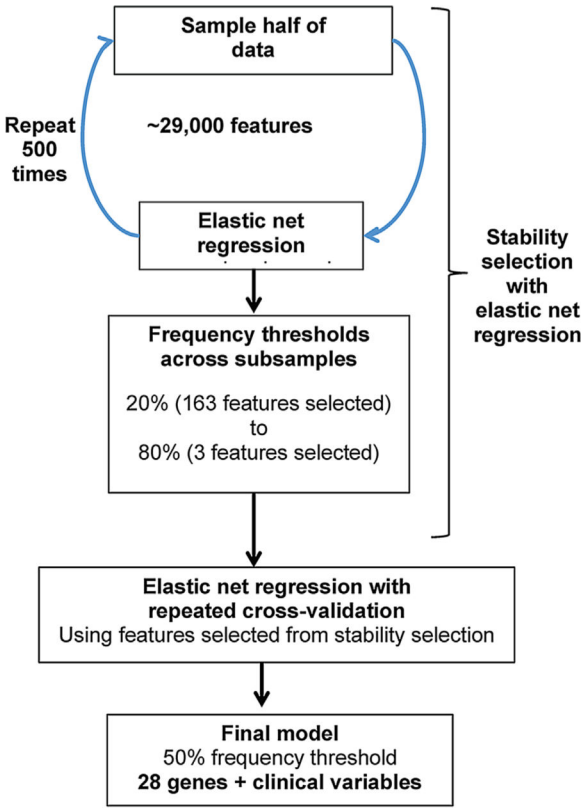

Fig. 1.
Analytical plan for predictive model development.

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TABLE I.

Clinical Characteristics of Localized Prostate Cancer Patients

	Controls (NED)	Cases (CR)	*P*-value
	n = 154	**n = 33**	
Age			
Median	64	66	0.056
IQR	56–69	62–71	
Range	44–82	45–76	
PSA before surgery (ng/ml)			
Median	7.16	7.4	0.17
IQR	4.81–10.40	5.60–13.0	
Range	1.1–120	2.2–65.80	
Pathologic Gleason score			
≤6	56 (36)	5 (15)	0.01
(3 + 4) or (2 + 5)	60 (39)	11 (33)	
(4 + 3) or (5 + 2)	14 (9)	5 (15)	
8–10	24 (16)	12 (36)	
Surgical margin status			
Negative	119 (77)	24 (73)	0.651
Positive	35 (23)	9 (27)	
Race/ethnicity			
Non-Hispanic White	137 (89)	29 (88)	0.255
Hispanic	12 (8)	1 (3)	
African-American	4 (3)	2 (6)	
Asian/PI	1 (1)	1 (3)	
Clinical stage			
cT1	105 (68)	17 (52)	0.089
cT2	48 (31)	15 (45)	
cT3	1 (1)	1 (3)	
Pathologic stage			
T2a	10 (6)	3 (9)	0.819

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	Controls (NED)	Cases (CR)	*P*-value
	n = 154	**n = 33**	
T2b	9 (6)	1 (3)	
T2c	134 (87)	29 (88)	
T2 with unknown laterality	1	0	
Prostatectomy year			
07/1988–07/1994	56 (37)	18 (55)	0.093
07/1994–03/2005	90 (59)	13 (39)	
03/2005–06/2008	6 (4)	2 (6)	
D'Amico risk groups (Those with available clinical data: Gleason, stage, PSA)			
Low	50 (40)	2 (8)	<0.001
Intermediate	60 (48)	11 (42)	
High	15 (12)	13 (50)	
Neoadjuvant hormonal therapy			
No	148 (96)	25 (76)	0.001
Yes	6 (4)	8 (24)	
Radiation therapy			
No	135 (88)	26 (79)	0.177
Yes	19 (12)	7 (21)	
Adjuvant hormone therapy			
No	151 (98)	33 (100)	
Yes	3 (2)	0 (0)	
Median follow-up time (IQR)	9.55 (6.61–15.25)	5.83 (4.18–8.69)	

NED, patients with "no evidence of disease" after radical prostatectomy; CR, patients with clinical recurrence after radical prostatectomy.

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TABLE II.

DEGs Identified as Part of Enriched Pathways Identified With Gene Ontology and KEGG

Gene	Main functions	Reported role in PCa	Reported in other cancers	Enrichment category	Expression in CR compared to NED	References
ACADSB	Fatty acid metabolism, mitochondrial localization, determines vitamin D levels			Metabolic (KEGG)	Lower	[82]
ADI1	Methionine salvage	Progression		Metabolic (KEGG)	Higher	[35–37]
AGPAT2	Phospholipid synthesis		Osteosarcoma	Metabolic (KEGG)	Higher	[64]
ALDH1A2	Retinoic acid synthesis			Metabolic (KEGG)	Lower	[83]
ANO7	Ion channel transporter, cell–cell interaction, glucose transport	Grade		Prostatic neoplasia (DE)	Lower	[39,40]
ARHGEF6	G protein			Actin/cytoskeleton reg. (KEGG)	Higher	
BAIAP2	Angiogenesis inhibitor, insulin receptor TK, filopodia formation, cell mobility, cancer cell growth			Actin/cytoskeleton regulation (KEGG)	Lower	[84]
BMPR2	BMP receptor, cell invasion	Cell invasion		Growth factor binding (GO)	Higher	[85]
CDKN2B	Cell proliferation		Several cancers	Pathways in cancer (KEGG)	Higher	[86,87]
CHPT1	Phosphatidylcholine metabolism		Breast	Metabolic (KEGG)	Higher	[65]
COLIA2	Collagen chain, cell proliferation and cell migration		Bladder	Growth factor binding (GO)	Higher	[60]
CTBP2	Transcriptional repressor, cell proliferation	Progression, cell proliferatio	Breast, esophagus, ovarian	Oxidoreductase activity (GO); pathways in cancer (KEGG); Prostatic neoplasm (DE)	Lower	[45–47]
CYB5A	Metabolism ferric Hb metabolism, autophagy		Pancreatic	Oxidoreductase activity (GO)	Lower	[55]
DCXR	Glucose metabolism	Biomarker	Liver, melanoma	Oxidoreductase activity (GO); metabolic (KEGG)	Lower	[48–50]
DECR1	Fatty acid metabolism, mitochondrial localization			Binding (GO)	Lower	
DUOX1	Hydrogen peroxide producer, cell migration, antimicrobial defense			Binding (GO)	Higher	[59]
ESM1	Endothelial cells factor		Breast and gastric	Growth factor binding (GO)	Higher	[61,88]
FMO5	Trimethylamine metabolism, determines mtDNA levels			Binding (GO)	Lower	[44]
GLDC	Glycine metabolism, mitochondrial localization		Lung	Metabolic (KEGG)	Higher	[66]
GRHPR	Pyruvate metabolism		Liver	Oxidoreductase activity (GO); binding (GO); metabolic (KEGG)	Lower	[56]
LAMA3	Base membrane formation, cell migration		Gastric	Pathways in cancer (KEGG)	Higher	[89]
LDAH	Cholesterol homeostasis	Risk		Prostatic neoplasia (DE)	Lower	[53,54]

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Gene	Main functions	Reported role in PCa	Reported in other cancers	Enrichment category	Expression in CR compared to NED	References
MAT2B	SAM biosynthesis, growth, tumorigenesis			Oxidoreductase activity (GO); metabolic (KEGG)	Higher	[58]
ME2	Metabolism, mitochondrial localization		Melanoma	Oxidoreductase activity (GO)	Lower	[57]
MYH9	Cytokinesis, cell motility, cell shape, cell migration		Head and neck	Actin/cytoskeleton reg. (KEGG)	Higher	[69]
NADSYN1	Metabolism (NAD synthesis, calcium), determines VitD levels			Metabolic (KEGG)	Lower	[90,91]
NRP1	Cell migration, cell survival, angiogenesis	Relapse	Bladder	Growth factor binding (GO)	Higher	[51,52]
PIAS4	Autophagy		Pancreas	Pathways in cancer(KEGG)	Higher	[92,93]
REPS2	Cell proliferation, vesicular trafficking, endocytosis	Progression		Prostatic neoplasia (DE)	Lower	[41–43]
RHBDF2	Protease		Ovarian	Growth factor binding (GO)	Higher	[63]
SRM	Spermidine synthesis, cell growth			Metabolic (KEGG)	Lower	
SYNJ1	Synaptic transmissions, membrane trafficking			Metabolic (KEGG)	Higher	[94]
TMSB4X	Cell proliferation, cell migration, differentiation, anti-apoptotic, cancer stem cells		Glioblastoma, CRC, breast, ovarian, lung, kidney, uterine	Actin/cytoskeleton reg. (KEGG)	Lower	[70–72]

DEG, differentially expressed gene; CR, clinical recurrence patients; NED, no evidence of disease patients; CRC, colorectal cancer.

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TABLE III.

Genes Included in the Prostate Cancer Recurrence Predictive Model

Rank	Gene symbol	Gene name	Gene function	Cytogenetic band	Fold change (CR:NED)	FDR adjusted P-value	Relevance to PCa and other cancers	References
4	*ABCC11*	ATP-binding cassette transporter, sub-family C, member 11	Transporter, drug resistance	16q12.1	2.387	0.0311	Breast and liver	[95,96]
13	*ABLIM1*	Actin binding LIM protein 1	Cytoskeleton, cell adhesion	10q25	−2.576	0.0248		[97]
3	*ADRA2C*	Alpha-2-adrenergic receptor	Adrenergic receptor, neurotransmitter release	4p16.3	−2.503	0.0928	Breast and colorectal	[98,99]
8	*CLEC4F*	C-type lectin domain family 4, member F	Glycolipids presentation (Kupffer cells)	2p13.3	2.962	0.0441		
6	*CPVL*	Carboxypeptidase, vitellogenic-like	Post-translational protein modification	7p15.1	2.213	0.2197		[100]
26	*DUOX1*	Dual oxidase 1	Hydrogen peroxide producer, cell migration, antimicrobial defense	15q15.3	3.143	0.0086		[59]
24	*DUOXA1*	Dual oxidase maturation factor 1	Hydrogen peroxide producer, DU0X1 activator, cell adhesion	15q21.1	2.511	0.0423		[101]
14	*EDARADD*	EDAR-associated death domain	Death domain containing protein	1q24.3	3.013	0.0136		
17	*F10*	Coagulation factor X	Coagulation factor	13q34	−2.099	0.2283		
19	*GLDC*	Glycine dehydrogenase	Metabolism (glycine), mitochondrial localization	9p22	3.147	0.0430	Lung	[66]
15	*GPR81*	G protein-coupled receptor-81	Lactate receptor, g-protein	12q24.31	−2.851	0.0193	Multiple	[102]
18	*KCNA3*	Potassium voltage-gated channel, shaker-related subfamily, member 3	Potassium channel	1p13.3	2.375	0.0774	Prostate	[103]
20	*KCNQ2*	Potassium voltage-gated channel, KQT-like subfamily, member 2	Potassium channel	20q13.3	−2.481	0.1202		
25	*LDAH*	Chromosome 2 open reading frame 43	Metabolism (cholesterol)	2p24.1	−2.623	0.0338	Prostate	[53,54]
23	*MB*	Myoglobin	Oxygen storage and diffusion	22q13.1	−2.15	0.1821	Breast	[104]
5	*MMP11*	Matrix metalloproteinase-11	Extracellular matrix breakdown	22q11.23	3.422	0.0006	Prostate and breast	[105–107]
16	*MYBPC1*	Myosin binding protein C	Myosin binding, muscle	12q23.2	−2.754	0.0020		
1	*NKX2–1*	NK2 homeobox 1	Thyroid function, morphogenesis	14q13	4.279	0.0248	Endometrial	[108]
28	*NPR3*	Natriuretic peptide receptor C/guanylate cyclase C	Natriuretic receptor	5p14-p13	−2.533	0.1410	Medulloblastoma	[109]

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Rank	Gene symbol	Gene name	Gene function	Cytogenetic band	Fold change (CR:NED)	FDR adjusted P-value	Relevance to PCa and other cancers	References
9	*OAS2*	2–5-oligoadenylate synthetase 2	viral infection response	12q24.2	−2.369	0.0971	Prostate	[110]
27	*PCA3*	Prostate cancer antigen 3	LncRNA	9q21.2	−2.019	0.2026	Prostate	[82]
12	*PCBP3*	Poly(rc) binding protein 3	Post-transcriptional activator	21q22.3	2.093	0.1432		
10	*PGC*	Progastricsin	Digestive enzyme	6p21.1	−3.937	0.0181	Prostate, gastric	[111,112]
21	*RAPGEF1*	Rap guanine nucleotide exchange factor (GEF) 1	Apoptosis, cell transformation	9q34.3	2.249	0.1536	GI and ovarian	[113,114]
22	*TUBB2B*	Tubulin, beta 2B class IIb	Microtubules	6p25	2.181	0.1354		
2	*UPK1A*	Uroplakin 1A	Growth, motility	19q13.3	2.58	0.0928	Gastric, CRC, esophageal, bladder	[115–117]
11	*UPK3B*	Uroplakin 3B	Apical plaques in urothelium	7q11.2	2.245	0.0811		
7	*ZYG11A*	Zyg-11 family member A, cell cycle regulator	Cell cycle	1p32.3	4.314	0.0002		

DEG, differentially expressed gene; CR, clinical recurrence patients; NED, no evidence of disease patients; FDR, false discovery rate; GI, gastrointestinal; CRC, colorectal cancer.

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TABLE IV.

In Silico Validation of 28-Gene Signature Using Three External Datasets

	MC	MSKCC	EMC
Tissue used for gene expression and clinical outcomes	333 PM (NED + BCR) versus 212 PM (CR)	131 PM versus 19 tissues from MET lesions	39 PM (non-CR) versus 9 PM (CR)
	AUC (95%CI)	AUC (95%CI)	AUC (95%CI)
USC 28 gene model + clinical variables	0.75 (0.72–0.77)	0.90 (0.86–0.94)	0.82 (0.74–0.91)
Clinical variables only *	0.72 (0.70–0.74)	0.86 (0.82–0.91)	0.76 (0.67–0.85)

PM, primary tumors; NED, no evidence of disease (no recurrence patients); CR, clinical recurrence; MET, metastasis tissue; MC, Mayo Clinic; MSKCC, Memorial Sloan-Kettering Cancer Center; EMC, Erasmus Medical Center; AUC, area under the curve; CI, confidence interval.

* Clinical variables in model: MC—Gleason score only; MSKCC—age at diagnosis, race/ethnicity, neo-adjuvant treatment, and adjuvant treatment for all patients (no missing data); EMC—pathologic stage and Gleason score (no missing data).

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