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(54) Title: GENE-BASED PREDICTION OF PSA RECURRENCE FOR CLINICALLY LOCALIZED PROSTATE CANCER PATIENTS

(57) Abrégé/Abstract:
Disclosed are methods, devices and kits for determining the likelihood of recurrence of prostate cancer using the expression levels of preferably three-gene classifier. The methods, devices and kits can be used independent of many nomograms currently in use or to improve the overall performance of such nomograms.

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GENE-BASED PREDICTION OF PSA RECURRENCE FOR CLINICALLY LOCALIZED PROSTATE CANCER PATIENTS

Background

Prior to undergoing radical prostatectomy or other aggressive treatments for prostate cancer, it is useful to know with as much accuracy as possible whether the procedure is likely to be curative. Typically, a physician may provide responses to a patient's request for prognostic information by declining to make specific predictions, or providing overall averages, or providing a subjective assessment or assigning the patient to a defined risk group (such as a high or low risk group) based on a model. Providing the most accurate assessment is almost always the proper response. To provide the best possible assessment, since the pathologic stage of cancer correlates with the probability of recurrence after surgery or treatment, many efforts have been made to predict the final pathologic stage or outcome in prostate cancer patients. To this end, many nomograms, algorithms and markers have been employed with the Kattan Nomogram being the most successful. Preferably, the concordance index is used to select a particular strategy from the considered models/nomograms/algorithms and the like. The Kattan nomogram is described in US5993388 and US6409664.

An aggressive therapy for the treatment of clinically localized prostate cancer is radical prostatectomy. Unfortunately, many men treated with radical prostatectomy later experience progression of their disease. Starting with an increase in serum PSA, which indicates recurrence, the cancer returns in many men months or years following surgery. Early identification, prior to detectable PSA, of men likely to ultimately experience recurrence would be useful in considering additional treatments while preserving quality of life. Further, accurate estimation of a likelihood of recurrence will also be useful in clinical

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trials to identify candidates for control groups or for an investigational treatment of interest.

Increased accuracy in the classification of newly diagnosed clinically localized prostate cancers is needed if treatment is to be better tailored to this subgroup of patients. As in other cancers, a number of molecular markers and gene signatures of phenotype and prognosis have been developed recently for prostate cancer. These have provided some significant insights into the existence of distinct classes of aggressive prostate cancer and a number of potential candidate gene markers. A clinically viable test that incorporates the expression values of a small number of gene markers is useful either standing alone or in conjunction with other tools such as the Kattan post-operative nomogram to assess risk of PSA recurrence is desirable.

Summary

In one aspect, a gene expression based assay is used to provide a prognosis of the recurrence of prostate cancer. The gene expression based assay is preferably based on a small number of genes. A three gene profile is preferred. In a preferred embodiment, the expression level of the three genes MYH11, SSBP1, and DPT provides prognostic information about the likelihood of prostate cancer recurrence. In alternative embodiments, the expression level of genes such as Filamin C, gamma, RAS like family 12 and Filamin A may be used. Other possible marker combinations include (i) Growth Arrest Specific 1, Smoothelin, Leiomodin 1 and Histone 1 H3d, and (ii) Sorbin and SH3 domain containing 1, PDZ and LIM domain 7, LIM and senescent cell-antigen like domains 2. Additional useful combinations of expression level based markers may be readily selected from Table 2.

In another aspect, a gene expression level based assay is used in conjunction with a clinical tool such as a nomogram that is based on multiple clinical indicators of prostate cancer prognosis.

In a preferred embodiment, a measurement of the expression of three genes and three control genes is determined. Preferably, the gene measurements are measured relative to the average of three control genes. These gene measurements along with the predictive probability from the Kattan nomogram are incorporated into a statistical model to generate a score reflecting the probability of recurrence or the risk of recurrence. This information can also be used to determine the likelihood of PSA levels rising following radical prostatectomy. This probability of recurrence can help patients make personalized decisions about their choice in therapy.

More specifically disclosed is a classifier for distinguishing between subjects in a high recurrence risk category of prostate cancer and subjects in a low recurrence risk category of prostate cancer. The preferred classifier comprises a classification procedure including measuring an expression level of at least two

diagnostic markers selected from TABLE 2 with each expression level measured relative to an average of the expression levels of two or more control markers. The expression level of the at least two diagnostic markers is then combined with a predictive probability based on a Kattan nomogram to generate the classifier, which may be also be in the form of a nomogram. Herein the Kattan nomogram is a composite measure, described, for instance in US Patent 6,409,664 and 5,993,388.

The Kattan nomogram is typically based on two or more members of a group consisting of PSA value prior to surgery (and prior to hormone therapy, if received), PSA at the time of prostatectomy, primary Gleason at surgery, secondary Gleason at surgery, prostatectomy Gleason Sum, the year of prostatectomy, the months disease free, whether or not surgical margins were positive, whether or not cancer was found in seminal vesicles, whether or not there was extra-capsular extension, whether or not cancer was found in lymph nodes (if any were removed), pre-radiotherapy PSA, radiation dose (if applicable), whether surgical margins were positive or negative (if applicable), whether there was seminal vesicle involvement (if applicable), whether there was lymph node involvement (if applicable), whether there was extra capsular involvement (if applicable), whether or not neo-adjuvant hormones were prescribed, whether or not neo-adjuvant radiation was prescribed.

The Kattan nomogram may also be a composite measure based on two or more members of a group consisting of pretreatment PSA level, combined Gleason grade, specimen Gleason sum, clinical stage, surgical margin status, prostatic capsular invasion maximum cancer length in a core, total length of cancer in the biopsy cores, percent of cores positive level, extraprostatic extension, level of extraprostatic extension, apoptotic index, percent of cancer in one or more cores, percent of high grade cancer in one or more cores, total tumor volume, zone of location of the cancer, presence of seminal vesicle invasion, type of seminal vesicle invasion, p53, Ki-67, p27, DNA ploidy status, lymph node status, and lymphovascular invasion.

A preferred classifier for distinguishing between subjects in a high recurrence risk category of prostate cancer and subjects in a low recurrence risk category of prostate cancer requires measuring the expression levels of diagnostic markers MYH11, SSBP1 and DPT.

Preferred control markers are TUBA, ALAS1 and ACTG1. Alternative preferred control markers are selected for relatively steady expression levels as detected by RT-PCR.

Another preferred classifier for distinguishing between subjects in a high recurrence risk category of prostate cancer and subjects in a low recurrence risk category of prostate cancer requires measuring the expression levels of at least one diagnostic marker selected from TABLE 2 in addition to MYH11, SSBP1 and DPT.

In another preferred embodiment two or more diagnostic markers are selected from the set of markers presented in TABLE 2. More specific sets of diagnostic markers include

Also disclosed is a method of predicting the recurrence of prostate cancer. The method includes the steps of (i) determining an expression level of DPT relative to a standard; (ii) determining expression level of at least one additional markers from TABLE 2; and (iii) transforming the expression level of DPT and the expression level of at least one additional markers from TABLE 2 into a score corresponding to a probability of recurrence of prostate cancer. The method may alternatively, to the step of transforming the expression level of DPT and the expression level of at least one additional markers from TABLE 2, have a step of combining the expression level of DPT and the expression level of at least one additional markers from TABLE 2 with at least one additional indicator of prostate cancer recurrence to determine a composite score. Such a composite score reflects a likelihood of recurrence of prostate cancer.

Preferably, the expression level of DPT and the expression level of at least one additional markers from TABLE 2 is determined prior to at least one treatment selected from the group consisting of prostectomy, hormone therapy,

single agent chemotherapy, two agent chemotherapy and treatment with a farnesyl transferase inhibitor.

This disclosure also covers, without limitation, a kit for detecting an expression level of at least DPT, the kit comprising reagents and a first probe set comprising a first probe specifically recognizing DPT and a second probe specifically recognizing a second Marker selected from TABLE 2. The kit may include a device for converting the expression level of DPT and the expression level of at least one additional markers from TABLE 2 into an indicator of a likelihood of recurrence of prostate cancer. The device may be in the form of a nomogram. More specifically, a preferred kit is implemented as one or more members of the group consisting of a mechanical device, a graphical representation, and software instructions to implement a user interface for providing a representation of the indicator of the likelihood of recurrence of prostate cancer. In yet a further aspect of the invention a kit is provided containing reagents for conducting a measurement of three genes and three control genes from a prostate tumor. Instructions (optionally as computer code) are provided to enable the gene measurements to be normalized to the average of three control genes. These measures along with the predictive probability from a nomogram are incorporated into a statistical model that generates a probability of recurrence.

Description of the Figures

Figure 1A shows a comparison of the classifier disclosed herein with the nomogram using the c-index based on results from an independent test series of 157 patients. The c-index for the classifier was apparently higher than the c-index for the nomogram (0.77 vs. 0.67).

Figure 1B shows the correspondence between the 5-year predictive estimates on the test set and the actual probabilities of recurrence. The classifier demonstrated a good calibration across the spectrum of predictions for the test set as compared to an ideal predictor, while the 5-year nomogram displayed less accuracy in detecting the more aggressive cases.

Figure 2A shows a Kaplan-Meier analysis for PSA recurrence-free probability to illustrate the difference in time to PSA recurrence for the predicted low- and high- risk groups (HR 6.85, 95% CI = 3.77 to 12.43, P < .001,). At 5 years, the absolute difference in PSA recurrence between the two groups was 58% (75% vs. 17 %).

Figure 2B shows the classifier, used in Figure 2A, applied to patients with Gleason score of 6 or 7.

Figure 2C shows the classifier, used in Figure 2A, applied to patients exhibiting pathological stage pT2 or pT3a.

Figure 2D shows the classifier, used in Figure 2A, applied to patients with pre-operative PSA concentration ≤10 ng/mL or 10<PSA≤20 ng/mL.

Figure 2E shows the classifier, used in Figure 2A, applied to patients with positive or negative surgical margins.

Figure 3 shows the application of the cut-off based on each model's highest accuracy as applied these the test set.

Detailed Description

Nomograms are widely used to predict prostate cancer recurrence. The most widely used nomogram is the Kattan nomogram described in US Patent 6,409,664 and 5,993,388. For those who have not received surgical treatment for their prostate cancer, these nomograms incorporate the following information: most recent PSA (prostate specific antigen) value, primary and secondary Gleason grade, physician's assessment of clinical stage (using the 1992 or 1997 UICC system), radiation therapy dose that is recommended (if applicable), the number of positive cores found during biopsy, the number of negative cores found during biopsy, whether or not neo-adjuvant hormones had been prescribed, and whether or not neo-adjuvant radiation had been prescribed. For those for whom surgery was performed, the factors include: PSA value prior to surgery (and prior to hormone therapy, if received), PSA at the time of prostatectomy, primary Gleason at surgery, secondary Gleason at surgery, prostatectomy Gleason Sum, the year of prostatectomy, the months disease free, whether or not surgical margins were positive, whether or not cancer was found in seminal vesicles, whether or not there was extra-capsular extension, whether or not cancer was found in lymph nodes (if any were removed), pre-radiotherapy PSA, radiation dose (if applicable), whether surgical margins were positive or negative (if applicable), whether there was seminal vesicle involvement (if applicable), whether there was lymph node involvement (if applicable), whether there was extra capsular involvement (if applicable), whether or not neo-adjuvant hormones were prescribed, whether or not neo-adjuvant radiation was prescribed. This information is combined in a spreadsheet, for example, and a simple statistical treatment provides an analysis used to determine prognosis.

The methods and kits of this invention are most preferably used in conjunction with these nomograms and the information they provide. The method and kits of the invention also involve the detection of a group of genes.

Preferably, the expression level one of the genes in this group is measured relative to at least one of the control markers. More preferably the expression levels of three genes and three controls are measured. The most preferred genes are MYH11, SSBP1, and DPT. The preferred controls are ALAS1, TUBA, and ACTG1. Higher expression of the genes MYH11 or DPT indicates a stronger likelihood of remaining free of prostate cancer recurrence than if such over-expression is not seen as described in the examples. A higher expression level of SSBP1 indicates a stronger likelihood of recurrence. The nucleic acid sequences that correspond to the genes whose expression level is measured as well as the sequences used to measure such expression levels are referred to in this specification as Markers. Other sequences of interest include genes useful as assay controls such as ALAS1, TUBA, and ACTG1. Markers are detected with any of the methods used to detect gene expression; prefcrably these are amplification based methods such as PCR, its variants, and alternative methods. Most preferably, RTPCR is used.

Nucleic acid probes or reporters specific for certain Markers are preferably used to detect the expression of the Marker gene in tumor tissue. Other biological fluids or tissues can be used including prostate tissue, urine, urethral washings, blood and blood components such as serum, ejaculate, and other samples from which prostate proteins could be expected. Any specimen containing a detectable amount of the relevant polynucleotide can be used.

One disclosed method includes contacting a target cell containing a Marker with a reagent that binds to the nucleic acid. The target cell component is a nucleic acid such as RNA. The reagents preferably include probes and primers such to amplify and detect the target sequence. For example, the reagents can include priming sequences combined with or bonded to their own reporter segments such as those referred to as Scorpion reagents or Scorpion reporters and described in US Patents 6,326,145 and 6,270,967 to Whitcombe et. al. Though they are not the same, the terms "primers" and "priming sequences" may be used in this specification to

refer to molecules or portions of molecules that prime the amplification of nucleic acid sequences.

Preferred primers are capable of initiating synthesis of a primer extension product, which is substantially complementary to a polymorphic locus strand. The primers and/or probes may be prepared using any suitable method including automated methods. Environmental conditions conducive to synthesis include the presence of nucleoside triphosphates and an agent for polymerization, such as DNA polymerase, and a suitable temperature and pH. The priming segment of the primer or priming sequence is preferably single stranded for maximum efficiency in amplification, but may be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent for polymerization.

Preferred primers are most preferably eight or more deoxyribonucleotides or ribonucleotides. The exact length of primer will depend on factors such as temperature, buffer, and nucleotide composition. The oligonucleotide primers most preferably contain about 12-20 nucleotides although they may contain more or fewer nucleotides.

When complementary strands of nucleic acid or acids are separated, regardless of whether the nucleic acid was originally double or single stranded, the separated strands are ready to be used as a template for the synthesis of additional nucleic acid strands. This synthesis is performed under conditions allowing hybridization of primers to templates to occur. Generally synthesis occurs in a buffered aqueous solution, preferably at a pH of 7-9, most preferably about 8. A molar excess (for genomic nucleic acid, usually about 108:1, primer: template) of the two oligonucleotide primers is preferably added to the buffer containing the separated template strands. The amount of complementary strand may not be known if the process of the invention is used for diagnostic applications, so the amount of primer relative to the amount of complementary strand cannot always be determined with certainty. As a practical matter,

however, the amount of primer added will generally be in molar excess over the amount of complementary strand (template) when the sequence to be amplified is contained in a mixture of complicated long-chain nucleic acid strands. A large molar excess is preferred to improve the efficiency of the process.

The agent for polymerization may be any compound or system that will function to accomplish the synthesis of primer extension products, preferably enzymes. Suitable enzymes for this purpose include, for example, E. coli DNA polymerase 1, Klenow fragment of E. coli DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, polymerase mutants, reverse transcriptase, and other enzymes, including heat-stable enzymes (e.g., those enzymes which perform primer extension after being subjected to temperatures sufficiently elevated to cause denaturating). A preferred agent is Taq polymerase. Suitable enzymes will facilitate combination of the nucleotides in the proper manner to form the primer extension products complementary to each locus nucleic acid strand. Generally, the synthesis will be initiated at the 3' end of each primer and proceed in the 5' direction along the template strand, until synthesis terminates, producing molecules of different lengths. There may be agents for polymerization, however, which initiate synthesis at the 5' end and proceed in the other direction, using the same process as described above.

In another aspect of the invention an expression ratio is used. Establishing a ratio between the amount of amplified Marker attained and the amount of amplified reference Marker or control Marker region amplified can do this. This can be done using quantitative real-time PCR. Ratios can be plugged into a statistical model to determine a likelihood of prostate cancer recurrence.

The kits of the invention can be configured with a variety of components, preferably such that they all contain at least one primer or probe or a detection molecule (e.g., Scorpion reporter). In one embodiment, the kit includes reagents for amplifying and detecting Marker segments. Optionally, the kit includes sample preparation reagents and /or articles (e.g., tubes) to extract nucleic acids from samples.

In a preferred kit, reagents necessary for RTPCR are included such as, a corresponding PCR primer set, a thermostable polymerase, such as Taq polymerase, and a suitable detection reagent(s) such as hydrolysis probe or molecular beacon. In optionally preferred kits, detection reagents are Scorpion reporters or reagents. A single dye primer or a fluorescent dye specific to double-stranded DNA such as ethidium bromide can also be used. Additional materials in the kit may include: suitable reaction tubes or vials, a barrier composition, typically a wax bead, optionally including magnesium; necessary buffers and reagents such as dNTPs; control nucleic acid (s) and/or any additional buffers, compounds, co-factors, ionic constituents, proteins and enzymes, polymers, and the like. Optionally, the kits include nucleic acid extraction reagents and materials.

In preferred kit of the invention, instructions to conduct the assay on patients with prostate samples are provided. It is most preferred that an article encoded with computer instructions for preparing a prediction from a Cox Proportional Hazard analysis or other statistical comparator is provided. The instructions are loaded into a computer such as a general purpose computer such that when the values of the gene analysis and, optionally, the Kattan parameters are input into the program, the computer provides as output the likelihood of recurrence (high versus low or a numerical indicator).

In a preferred kit of the invention, mechanical implementations of a nomogram may be also provided. Such implementations may use paper based components or moving mechanical parts to allow consideration of the individual variables used by the nomogram.

Examples.

EXAMPLE 1

Purpose: Accurate estimates of the risk of recurrence are needed for the optimal management of patients with clinically localized prostate cancer. A nomogram and novel molecular predictors were combined into a new prognostic model of prostate specific antigen (PSA) recurrence.

This study was designed to identify genes that correlate with PSA recurrence in patients with clinically localized prostate cancer with the goal of developing an accurate predictive classifier that can be readily applied in current routine clinical practice for management of patients with organ-confined disease. Here we report the development of a clinically viable test that incorporates the expression values of three novel gene markers, measurable by RTPCR, and the Kattan post-operative nomogram (11), a widely used tool in the clinical management of prostate cancer, to assess risk of PSA recurrence. Finally, we show that this new classifier provides improved accuracy compared with the Kattan nomogram for predicting biochemical recurrence in this lower risk patient population.

Materials and Methods: Gene expression profiles from formalin-fixed, paraffin-embedded (FFPE) localized prostate cancer tissues were analyzed to identify genes associated with PSA recurrence. The profiles of the identified markers were reproduced by reverse-transcriptase-polymerase-chain-reaction (RTPCR). The RTPCR profiles from three of these genes, along with the output from the Kattan post-operative nomogram, were used to produce a predictive model of PSA recurrence.

Results: After variable selection, a model of PSA recurrence was built that combined expression values of three genes and the post-operative nomogram. The 3-gene plus nomogram model predicted 5-year PSA recurrence with a concordance index (c-index) of 0.77 in a validation set compared to a c-index of 0.67 for the nomogram. This model identified a subgroup of patients that were at high risk for recurrence which were not identified by the nomogram.

Conclusions: This new gene-based classifier has superior predictive power when compared against the 5-year nomogram to assess risk of PSA recurrence in patients with organ-confined prostate cancer. This classifier should provide a more accurate stratification of patients into high and low risk groups for treatment decisions and adjuvant clinical trials.

MATERIALS AND METHODS

Patients and tumor samples

Patient information was obtained from the St. Vincent's Campus Prostate Cancer Group (SVCPCG) database (Human Research Ethics Committee Approval H00/088). From January 1990 to December 2001, 960 patients were treated for prostate cancer with radical prostatectomy (RP) with no preoperative therapy at St Vincent's Hospital, Sydney. The subgroup of 316 consecutive patients with clinically localized disease assessed in the current study are those patients of the 960 for which the pathological stage ranged from pT2A to pT3A; the minimum follow-up for censored patients was five years; RP was the primary treatment; and tissue blocks could be accessed from the RP specimens for use in gene expression profiling experiments. The date of PSA recurrence was defined as the date of the first increase in serum PSA $\geq$ 0.2 ng/mL after RP. These patients were randomly split into training and test sets. The test set was used solely for validation purposes. Differences in the distribution of the clinical variables between the training and test sets were evaluated by either a t-test, log-rank, or Chi-square test depending on whether the variable was continuous, time-to-event, or categorical. All statistical tests were two-sided and significance was defined as $p < 0.05$.

Gene expression profiling

Six μm sections from each of the FFPE tissue blocks were submitted to pathology review (JGK) and macrodissected to ensure > 30% malignant epithelium was used for total RNA extraction using the High Pure RNA Paraffin kit (Roche Diagnostics, Indianapolis, IN).

Gene expression profiling experiments were performed on all total RNA specimens in the training series with a 1200 gene custom designed DNA-mediated annealing, selection, ligation and extension microarray (DASL) (Illumina, San Diego, CA)(22). Both the gene expression profiles and the design of the DASL array can be accessed in the GEO. Three control genes, ALAS1, TUBA and ACTG1, were selected for the

microarray and RTPCR analysis based on earlier studies in prostate cancer (12, 23).

Significance Analysis of Microarrays (SAM) analysis using the survival mode was used to measure prognostic significance for each probe on the array (24-26). The probes were ranked by the absolute value of the test statistic. False discovery rates were calculated by data permutation.

RTPCR assays were designed for the top-ranked prognostic marker candidates (n=30), including all genes under the lowest reported false discovery rates for both increasing and decreasing probes. Genes with a poor Pearson correlation (<0.4) between the array and RTPCR data among the training samples were excluded from further analysis.

Construction of the prognostic model

Using the Memorial Sloan Kettering Cancer Centre on-line calculator (http://www.mskcc.org/mskcc/html/10088.cfm), the five-year nomogram recurrence score was calculated for each individual patient using the post-operative historical model. To select variables for a multivariate model, the delta CT values of the candidate genes from RTPCR as well as the predicted probabilities from the nomogram were processed by the L1 regularization path algorithm using the training samples (25). By cross-validating the training series using the path algorithm to set different limits on the potential for over-fitting the Cox model, the signature with the least error was selected. The final predictive model for deployment was built by fitting these selected variables to the training set using a Cox proportional-hazards model.

A cut-off for high and low risk stratification of the model, and a cut-off for the nomogram were both chosen under the assumption that the costs for false positives and false negatives are equivalent. Under this assumption, a cut-off giving the highest training set accuracy (defined by the total number of correctly classified patients) was chosen.

Validation of the prognostic model

To evaluate the accuracy of predictive prognostic models with respect to the actual freedom from recurrence in the test set, a calibration curve was generated from the predicted 5-year recurrence-free probability estimated by Cox proportional-hazards regression and the Kaplan-Meier estimates of the actual recurrence-free probability at 5-years (24, 27). The performance of the final prognostic model in the test set was assessed by Kaplan-Meier curves and hazard ratios by stratifying the test set patients into a low risk and high risk group based on the pre-selected cut-off from the training set. All statistical analyses were performed in R, version 2.5.0 (www.r-project.org).

RESULTS

Patient characteristics

Total RNA was isolated from 316 prostatectomy FFPE tissues and 20 samples were excluded due to RNA degradation. The clinical and pathological characteristics of patients included in the training and test sets are summarized in Table 1. Median follow-up was 72 months, and median time from radical prostatectomy to biochemical recurrence was 34 months among those who recurred. Ninety-eight of 296 patients developed recurrence, including 74 patients who developed recurrence within 5 years of surgery. The training series consisted of 138 patients with the remaining 158 set aside for the test set. There was no statistically significant difference in the clinicopathological characteristics between these two sets of patients (Table 1).

Gene expression and univariate analysis

The RNA samples from the training set of 138 patients were analysed by DASL array. The permutation of the SAM algorithm revealed false discovery rates of 0% for 20 genes on the DASL array. The top 30 genes as ranked by the score value from the SAM analysis had a false discovery rate of 6.8%. These 30 genes were then assessed by RTPCR analysis using the same training set. Six of the 30 selected genes displayed a correlation between DASL and RTPCR of less than 0.4 and thus were removed from further analysis. The effect of each gene on

recurrence free probability was measured by Cox regression. The hazard ratio quantified the relative risk of PSA recurrence for each increase of 1 normalized CT. The hazard ratio and P value are recorded for both the training and test sets (Table 2). Twenty-three of 24 markers (except for marker HIST1H3D) continued to have a significant association to recurrence in the test set. In the same analysis, a 5-year postoperative nomogram was also a significant predictor of PSA recurrence in both the training and test sets (P value of .001 and .005, respectively).

Example 2:

Further variable selection was performed on the RTPCR training set of Example 1 to build a multivariate prognostic classifier. Four variables were selected by the L1 Regularization algorithm: 3 genes (DPT, SSBP1 and MYH11) and the 5-year nomogram. These 4 variables were then modeled on the training set using Cox regression analysis.

Classifier validation and survival analysis

When testing the prognostic model on an independent test series of 157 patients, the c-index for the classifier was apparently higher than the c-index for the nomogram (0.77 vs. 0.67) (Figure 1A). The nomogram performance was consistent with published studies (c-index of 0.72) when tested on a consecutive prostate cancer patient cohort consisting of 960 patients from the same institution that was not limited to organ-confined disease (Figure 1A). We then used calibration curves to measure how close the 5-year predictive estimates on the test set were to the actual probabilities of recurrence. The classifier demonstrated a good calibration across the spectrum of predictions for the test set as compared to an ideal predictor, while the 5-year nomogram displayed less accuracy in detecting the more aggressive cases (Figure 1B).

The cut-off from the training set was used to place test set patients into either a high- or low- risk group. Kaplan-Meier curves were generated for the test set samples by stratifying the patients into a low risk and high risk group based on a cutoff from the training set that produced the highest accuracy on the training

samples. A calibration curve was generated from the predicted 5-year disease-free survival of the test set and the Kaplan-Meier estimates at 5¬years based on cuts in the predicted probability at 0.3,0.5,0.7 and 0.9. The Kaplan-Meier analysis for PSA recurrence-free probability showed a highly significant difference in time to PSA recurrence for the predicted low- and high- risk groups (HR 6.85, 95% CI = 3.77 to 12.43, P < .001, Figure 2A). At 5 years, the absolute difference in PSA recurrence between the two groups was 58% (75% vs. 17 %). In addition, the classifier also represented a strong prognostic factor for PSA recurrence in the following subgroups of patients: Gleason score 6 or 7 (Figure 2B), pathological stage pT2 or pT3a (Figure 2C), pre-operative PSA concentration ≤10 ng/mL or 10<PSA≤20 ng/mL (Figure 2D), and positive or negative surgical margins (Figure 2E).

The clinical and pathological characteristics of patients included in the training and test sets are summarized in Table 1. Median follow-up was 72 months. Mean and median times from radical prostatectomy to biochemical recurrence were 40 and 34 months, respectively. Ninety-eight of 296 (33%) patients developed recurrence, including 74 (25%) patients who developed recurrence within 5 years of surgery.

Application of an improved prognostic model

In order to evaluate the potential impact of the model on patient management, we compared the accuracy of prognostic stratification using the classifier compared to the 5-year post-operative nomogram on the test cohort. We used the cut-off based on each model's highest accuracy and then applied these to the test set (Figure 3). Within the 157 test set patients, 136 predicted as low risk by the nomogram had a recurrence rate of 23.5% (32/136). In comparison, when applied to this group, the classifier identified 14 patients as having a "high risk" of recurrence including 12 patients that had a documented recurrence (86%). Of the 122 patients for whom the classifier conferred "low risk" status, 20 patients (16.4%) experienced a PSA recurrence. Conversely, none of the 11 patients that were predicted as the "high risk" by the nomogram but "low risk" by the classifier

had a documented recurrence. Thus the classifier conferred additional prognostic information to that provided by the postoperative nomogram on this series of prostate cancer patients.

Example 3:

RTPCR assays were designed for the candidate markers and the three (3) control genes. Multiple PCR primers and probes were designed for the markers. CT values were normalized to the average of 3 control genes.

Each normalized gene was plotted against the DASL signal values. Total RNA was reverse 15 transcribed, and pre-amplified with the gene-specific primers. Pre-amplified cDNA was then quantified using ABI PRISM® 7900 sequence detection system (APPLIED BIOSYSTEMS).

DPT-1232 3096:

Forward primer GGGTTGGAAGGATTTCCTGAA (SEQ ID NO 1)

Reverse primer CCCTGCACTCATTTTCCTTACTG (SEQ ID NO2)

Probe 5'Fam-3'MGB labeled probe TAGAAGACAAACGTTAGCATAC (SEQ ID NO 3)

MYH11-5893 460:

Forward primer GCACTCAAGAGCAAGCTCAGAG (SEQ ID NO 4)

Reverse primer TCGTTTCCTCGCCTGGTG (SEQ ID NO 5)

Probe 5'Fam-3'MGB labeled probe AGGAAACTTCGCAGTGAT (SEQ ID NO 6)

SSBP1-291 2990:

Forward primer AGTTTACCAACTGGGTGATGTCAG (SEQ ID NO 7)

Reverse primer 5'Fam-3'MGB TTGATATGCCACGTCTCTGAGG (SEQ ID NO 8)

labeled probe ATGGCACAGAATATCAG (SEQ ID NO 9)

Example 4:

Also identified were additional panels, which while suboptimal compared to the preferred panel, are also useful in their own right. Each of the alternative panels can be used as a stand alone panel or in combination with the Kattan

nomogram, the performance of which is improved by each of the alternative panels. The arrow symbol indicates the c-index corresponding to the model.

Alt1:

FLNC+RASL12+MYLK+FLNA+NOM_H5 –> 0.71

FLNC+RASL12+MYLK+FLNA –> 0.67

Alt2:

GAS1+SMTN+LMOD1+HIST1H3D+NOM_H5 –> 0.74

GAS1+SMTN+LMOD1+HIST1H3D –> 0.71

Alt3:

SORBS1+PDLIM7+LIMS2+MT1X+NOM_H5 –> 0.73

SORBS1+PDLIM7+LIMS2+MT1X –> 0.69

Example 5:

Also identified were probes and primer pairs for the remaining markers. They are presented in Table 3.

Example 6:

An alternative set of primers and probes were also developed for MYH11, DPT and SSBP1 as well as ALAS, ACTG and TUBA. The results were at least consistent or superior compared to the primer and probe sets of Example 3. They alternative primer and probes are:

DPT558F TGCAGTGGAAAGGGATCGC (SEQ ID NO 10)

DPT640R CCCAGATTTGGTATGTGGCA (SEQ ID NO 11)

DPT588P_FAM CATAATGTGCCGGATGACTGAATA (SEQ ID NO 12)

MYH 5895 F GCACTCAAGAGCAAGCTCAGA (SEQ ID NO 13)

MYH 5964R TAGAAGGAACGAAAGAGGTCTC (SEQ ID NO 14)

MYH 5925 P_Orange CCACAGGAAACTTCGCAGTGAT (SEQ ID NO 15)

SSBP1;281F GGGATAGTGAAGTTTACCAAC (SEQ ID NO 16)

SSBP1;359R TTGATATGCCACGTCTCTGA (SEQ ID NO 17)

SSBP1;312P (ORANGE) CAGTCAAAAGACAACATGGCACAG (SEQ ID NO 18)

TUBA 586 F TTCGCAAGCTGGCTGA (SEQ ID NO 19)

TUBA 666 R CATGAGCAGGGAGGTGAA (SEQ ID NO 20)

TUBA 639 P_Cy5 (Quasar) AGCTTTGGTGGGGGAACTGGTTCT (SEQ ID NO 21)

ALAS918F CAAGTGTCAGTCTGGTGCA (SEQ ID NO 22)

ALAS984R GTTGTTTCAAAGTGTCCATAAC (SEQ ID NO 23)

ALAS948P_FAM CTAGGAATGAGTCGCCACCCAC (SEQ ID NO 24)

ACTG 862 F AGCCTTCCTTCCTGGGTAT (SEQ ID NO 25)

ACTG 903 R TGATGGAGTTGAAGGTGGT (SEQ ID NO 26)

ACTG 882 P_Cy5 (Quasar) GAATCTTGCGGCATCCACGA (SEQ ID NO 27)

DISCUSSION:

The relatively low level of complexity of the disclosed classifiers is also important with the ability to measure expression of a small set of genes in FFPE tissue with a diagnostic-approved platform. These significant advances address some key factors affecting the likelihood of successfully implementing this predictive tool in a clinical diagnostic setting. The need for very small concentrations of RNA derived from FFPE tissue will also facilitate its potential long-term applicability to routine pathology specimens including preoperative transrectal biopsies.

This systematic assessment of prostate cancer-related gene expression correlates of PSA recurrence in order to develop a gene-based classifier of recurrence in clinically localized prostate cancer of potential broad clinical utility. The novel gene predictors were identified using a custom DASL array, a microarray platform that allows high-throughput gene expression profiling of RNA derived from FFPE tissues (22). A key component of this study was the design of the custom DASL microarray gene set that is based on gene markers

that were identified by re-analysis of published datasets (12, 13), in-house gene expression data (unpublished), and markers previously implicated in prostate cancer progression (14,16,17). This affords a degree of independent validation for those 30 genes that were most significant in this study. The 24-gene markers that correlated with PSA recurrence in the gene expression array analysis were further validated by RTPCR to produce a preferred 3-gene signature (DPT, MYH11 and SSBP1) that when combined with an established nomogram, resulted in a new classifier of PSA recurrence. This classifier was subsequently validated in an independent group of patients. DPT and MYH11 are novel prostate cancer prognostic markers while SSBP1 has previously been associated with aggressive prostate cancer (17).

An assessment of the value of this new classifier over a widely used nomogram for prostate cancer recurrence showed that the new classifier identified patients with both low- and high- risk of recurrence with much greater accuracy than the postoperative nomogram alone (9). The classifier presented here was also able to stratify patients within clinically relevant subgroups based on conventional clinicopathological parameters into high- and low risk- recurrence groups. Of note, the ability to stratify patients with Gleason 6 and 7 cancers represents a significant advance in predictive accuracy over current approaches. The use of PSA recurrence as a significant endpoint for prostate cancer has been disputed since only a proportion of patients who experience recurrence progress to clinically significant disease. These relationships will be more clearly defined as this cohort matures with data on metastases and death from PrCa. However, it is clear that the detection of a rising PSA post-prostatectomy is an important decision point when most physicians and patients consider further treatment options (5). In this context, the use of the 5-year nomogram to evaluate the potential impact of this classifier is valid with the majority of biochemical recurrences post-prostatectomy occurring within 5 years.

Of significance is the impact of this new classifier as a decision tool when considered against other published signatures and gene markers of molecular

phenotype and prognosis in prostate cancer (12-20). This study is unique in being developed specifically to aid prediction of risk of recurrence in prostate cancer patients with clinically localized disease, since these patients represent >80% of newly diagnosed cases of prostate cancer in the United States. While published signatures and gene markers have been identified from cohorts of patients representing the spectrum of pathological stages, both the training and test cohorts in this study were restricted to organ-confined prostate cancer. The importance of having concordance between the patient group used in the development of a predictive tool, with the anticipated target group is reinforced by the relatively low performance of the 5-year nomogram in this cohort of clinically localized patients compared with previous reports. On further analysis, this is likely due to the limitation of assessing only organ-confined cases in this study. When tested on our consecutive prostate cancer patient cohort from the same institution that was not limited to organ-confined disease, the nomogram performance was consistent with previous studies (9, 11).

The relatively low level of complexity of the classifier is also important. With the ability to measure expression of a small set of genes in FFPE tissue and the use of a platform that is approved for diagnostic testing in archival specimens, it is a significant advance as it addresses some key factors affecting the likelihood of successfully implementing this predictive tool to a clinical diagnostic setting. The requirement for low concentrations of RNA derived from FFPE tissue will also facilitate its potential long-term applicability to routine pathology specimens including preoperative transrectal biopsies. Further validation in external cohorts of both surgical and preoperative biopsies, including replicating the gene selection in biopsies, is now required to confirm the wider applicability of this classifier in the preoperative setting.

The implementation of an accurate predictive classifier for localized prostate cancer has important implications for patient management of early prostate cancer. Patients with localized disease and high-risk features are likely to benefit from adjuvant therapies including hormone, radiation and systemic

treatments and the benefits should be evaluated in treatment trials (7, 28, 29). Early phase clinical trials employing such agents are underway in the hormone-refractory setting but may ultimately be tested in localized prostate cancer as adjuvant therapies (30). Intrinsic to these studies is the accurate identification of high risk patients to ensure homogeneous patient groups8. Conversely, the improved identification of patients of low risk of recurrence will reduce the number of patients who are exposed to the morbidity of therapy as a result of the identification of increasing numbers of indolent cancers through PSA screening.

Finally, the development of an improved prognostic model for localized prostate cancer has the potential to facilitate better treatment decisions, either to forego treatment of indolent disease or offer adjuvant chemotherapy for men with high risk of recurrence.

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Table 1. Patient Characteristics of the Test and Training Cohorts.

Characteristic	*Training Cohort (no. of patients = 138)*	*Test Cohort (no. of patients = 158)*	*P Value*
Age, years			
<60	58	56	.50†
≥60	80	102	
Gleason Score			
≤6	57	75	.17*
7	64	72	
8-10	17	10	
Unknown	0	1	
Clinical Stage			
T1	56	67	.75*
T2	80	90	
T3	2	1	

pT Stage			
pT2a	4	13	.07*
pT2b	13	14	
pT2c	61	81	
pT3a	60	50	
PSA at diagnosis			
≤10	97	107	.55†
10<PSA≤20	34	43	
>20	7	8	
Extracapsular Extension			
Capsular Invasion	64	84	.13*
Focal	51	39	
Established	9	12	
None	14	23	
Margins			

Positive	53	70	.36*
Negative	85	88	
Adjuvant Treatment			
Yes	12	13	1.00*
No	126	145	
Outcome			
Disease-Free	89	109	.40‡
PSA recurrence	49	48	
Clinical (Local/Distant)	0	1	
Outcome at 5 Years			
Disease-Free	101	121	.47‡
PSA recurrence	37	36	
Clinical (Local/Distant)	0	1	

* The P value was calculated by the chi-square test.

† The P value was calculated by the t-test with the characteristic assessed as a continuous variable.

‡ The P value was calculated by the log-rank test.

Table 2. Cox regression for each tested RTPCR marker in the Test and Training cohorts.

MARKER	*Training Hazard Ratio†*	*Training P Value**	*Test Hazard Ratio †*	*Test P Value**	*DESCRIPTION*
ACTG2	1.6	< .001	1.35	< .001	Actin, gamma 2
CALD1	1.37	.007	1.44	.003	Caldesmon 1
CBX3	0.53	.05	0.64	.02	Chromobox homolog 3
DCHS1	1.52	.004	1.66	< .001	Dachsous 1
DKK3	1.53	.002	1.75	< .001	Dickkopf homolog 3
DPT	1.48	< .001	1.20	< .001	Dermatopontin
FLNA	1.31	.004	1.43	.005	Filamin A, alpha
FLNC	1.65	< .001	1.50	< .001	Filamin C, gamma
GAS1	1.43	< .001	1.59	< .001	Growth arrest-specific 1
GSN	1.63	.003	2.02	.001	Gelsolin
HIST1H3D	0.75	.008	0.89	.20	Histone 1, H3d

LIMS2	1.75	< .001	1.76	< .001	LIM and senescent cell antigen-like domains 2
LMOD1	1.80	< .001	1.57	< .001	Leiomodin 1
MT1X	1.56	.001	2.06	< .001	Metallothionein 1X
MYH11	1.68	< .001	1.29	< .001	Myosin, heavy polypeptide 11
MYLK	1.73	< .001	1.56	< .001	Myosin, light polypeptide kinase
PDLIM3	1.37	.003	1.33	.002	PDZ and LIM domain 3
PDLIM7	1.92	< .001	1.47	.01	PDZ and LIM domain 7
RASL12	1.84	< .001	2.09	< .001	RAS-like, family 12
SH3BGRL	1.32	.04	1.81	< .001	SH3 domain binding glutamic acid-rich protein like
SMTN	1.89	< .001	1.78	< .001	Smoothelin
SORBS1	1.68	< .001	1.40	.002	Sorbin and SH3 domain containing 1
SSBP1	0.34	.02	0.32	< .001	Single-stranded DNA binding protein 1

TNS1	1.88	< .001	1.70	.001	Tensin 1

* Cox regression p-value

† The hazard ratio is for each increase of 1 in the normalized CT value.

Table 3. Primer pairs and probes for the markers.

	SEQ ID	MARKER NAME	OLIGONUCLEOTIDE	5'-3' SEQUENCE
1	SEQ ID 28	ACTG1	5'Fam-3'MGB labeled probe	TTGCGGCATCCAC
	SEQ ID 29	ACTG1	Forward primer	CAGCCTTCCTTCCTGGGTATG
	SEQ ID 30	ACTG1	Reverse primer	CATGATGGAGTTGAAGGTGGTCT
2	SEQ ID 31	ACTG2	5'Fam-3'MGB labeled probe	CATGAGACAACCTACAATT
	SEQ ID 32	ACTG2	Forward primer	TTTATTGGCATGGAGTCCGC
	SEQ ID 33	ACTG2	Reverse primer	CCTTACGGATGTCAATGTCACACT
3	SEQ ID 34	ALAS1	5'Fam-3'MGB labeled probe	CAGTATGATCGTTTCTTTGAG
	SEQ ID 35	ALAS1	Forward primer	ATAACTTGCCAAAATCTGTTTCCACT
	SEQ ID 36	ALAS1	Reverse primer	AAACTCGATAGGTGTGGTCATTCTT
4	SEQ ID 37	CALD1	5'Fam-3'MGB labeled probe	ATGCCTGATGACCTATAA
	SEQ ID 38	CALD1	Forward primer	CATGGCAGATAGGTATCAATATGTT TTC
	SEQ ID 39	CALD1	Reverse primer	TCAACTCCTTCTAACAGTTCTAATCT CTCT
5	SEQ ID 40	CBX3	5'Fam-3'MGB labeled probe	ATTTGCCAGAGGTCTTGAT
	SEQ ID 41	CBX3	Forward primer	AAAGAGATGCTGCTGACAAACCA
	SEQ ID 42	CBX3	Reverse primer	CATCAATTCTCCACTGCTGTCTG
6	SEQ ID 43	DCHS1	5'Fam-3'MGB labeled probe	TGAACAGCTCAACAGGG

	SEQ ID 44	DCHS1	Forward primer	GCCGTGAGGCATTTGCA
	SEQ ID 45	DCHS1	Reverse primer	CACTCGCGCACGCAACT
7	SEQ ID 46	DKK3	5'Fam-3'MGB labeled probe	CAGACTGGACAAATGG
	SEQ ID 47	DKK3	Forward primer	CGAGAAATTCACAAGATAACCAACA
	SEQ ID 48	DKK3	Reverse primer	CTGCCTTCTTCGTCTCCCAC
8	SEQ ID 3	DPT	5'Fam-3'MGB labeled probe	TAGAAGACAAACGTTAGCATAC
	SEQ ID 1	DPT	Forward primer	GGGTTGGAAGGATTTCCTGAA
	SEQ ID 2	DPT	Reverse primer	CCCTGCACTCATTTTCCTTACTG
9	SEQ ID 49	FLNA	5'Fam-3'MGB labeled probe	ATGGCCCAAGGAC
	SEQ ID 50	FLNA	Forward primer	CAGCAAAGCAGGCAACAACAT
	SEQ ID 51	FLNA	Reverse primer	CGTGCTTCACCAGGATCTCC
10	SEQ ID 52	FLNC	5'Fam-3'MGB labeled probe	CAACCCCAGAGTTTTAAGGA
	SEQ ID 53	FLNC	Forward primer	GGTCTGGTCTCTCTGGTGGCT
	SEQ ID 54	FLNC	Reverse primer	TTCTCTGATTGTGCTTTCCTTTCC
11	SEQ ID 55	GAS1	5'Fam-3'MGB labeled probe	TATAGAATCCATTTGTCATCAGG
	SEQ ID 56	GAS1	Forward primer	ACTCACATCCATATTACACCTTTCCC
	SEQ ID 57	GAS1	Reverse primer	TAAATATAGCACACTTCACAATGGACTGT

12	SEQ ID 58	GSN	5'Fam-3'MGB labeled probe	CCGAGTTCCTCAAGGC
	SEQ ID 59	GSN	Forward primer	GCGGCCCAACAGCATG
	SEQ ID 60	GSN	Reverse primer	TGCAGGCCAGGCTCCTT
13	SEQ ID 61	HIST1H3D	5'Fam-3'MGB labeled probe	AAGTTCGCAATGGCTCGTA
	SEQ ID 62	HIST1H3D	Forward primer	CAAGGCCAAGGCAGGTTTTAG
	SEQ ID 63	HIST1H3D	Reverse primer	CACCCGTGGACTTGCGAG
14	SEQ ID 64	LIMS2	5'Fam-3'MGB labeled probe	TCCACACCCACAAGC
	SEQ ID 65	LIMS2	Forward primer	CACACTGAGCCAGCAAGTCCT
	SEQ ID 66	LIMS2	Reverse primer	TTCCGAAGGATGGAGGTGG
15	SEQ ID 67	LMOD1	5'Fam-3'MGB labeled probe	CTGAACTGTGAGTCCTGAT
	SEQ ID 68	LMOD1	Forward primer	GCTGTGCCCCACCTGTTG
	SEQ ID 69	LMOD1	Reverse primer	TAGAGTCCTCCAGGGAGCCC
16	SEQ ID 70	MT1X	5'Fam-3'MGB labeled probe	CTCGAAATGGACCCCAAC
	SEQ ID 71	MT1X	Forward primer	GATCGGGAACTCCTGCTTCTC
	SEQ ID 72	MT1X	Reverse primer	CAGGAGCCAACAGGCGAG
17	SEQ ID 6	MYH11	5'Fam-3'MGB labeled probe	AGGAAACTTCGCAGTGAT
	SEQ ID 4	MYH11	Forward primer	GCACTCAAGAGCAAGCTCAGAG

	SEQ ID 5	MYH11	Reverse primer	TCGTTTCCTCGCCTGGTG
18	SEQ ID 73	MYLK	5'Fam-3'MGB labeled probe	TCTGAAGAAGATGTGTCCCA
	SEQ ID 74	MYLK	Forward primer	CCAGCCCGCTCAATGC
	SEQ ID 75	MYLK	Reverse primer	CTCAGCAACAGCCTCAAGGAA
19	SEQ ID 76	PDLIM3	5'Fam-3'MGB labeled probe	GAAGATCACACCTTTTAATG
	SEQ ID 77	PDLIM3	Forward primer	GGATAATGGCAAGCCACTCATAA
	SEQ ID 78	PDLIM3	Reverse primer	TCTGTTTCTCTCCTTCTCTCTTCCA
20	SEQ ID 79	PDLIM7	5'Fam-3'MGB labeled probe	CTGAAGATGACCTGGCACG
	SEQ ID 80	PDLIM7	Forward primer	GAAGAAGATTACAGGCGAGATCATG
	SEQ ID 81	PDLIM7	Reverse primer	CAGGCAGCACAGGTAAAGCA
21	SEQ ID 82	RASL12	5'Fam-3'MGB labeled probe	CTTCCCGACCCACAGGCCAGCT
	SEQ ID 83	RASL12	Forward primer	ACCACATGCTTGCAGTCCTACA
	SEQ ID 84	RASL12	Reverse primer	AGTGGCCTGGAGCAAAAGTG
22	SEQ ID 85	SH3BGRL	5'Fam-3'MGB labeled probe	CTAGCAAAGAGATTAGACTTT
	SEQ ID 86	SH3BGRL	Forward primer	CATGAAGTGGGATGCCAAGTAA
	SEQ ID 87	SH3BGRL	Reverse primer	GATCGCCAACCTGTTTTATAAGAGT
23	SEQ ID 88	SMTN	5'Fam-3'MGB labeled probe	TTCACCTATGTGCAGTCG

	SEQ ID 89	SMTN	Forward primer	GCAAGAAGCCTGACCCCAA
	SEQ ID 90	SMTN	Reverse primer	TCGTGGCGTCGCAGGT
24	SEQ ID 91	SORBS1	5'Fam-3'MGB labeled probe	CTTTAATGGTGATACACAAGTAGA
	SEQ ID 92	SORBS1	Forward primer	CCAGTGCAGGTTTTGGAATATG
	SEQ ID 93	SORBS1	Reverse primer	TGATCCTCTCACCCTTTCTGAAG
25	SEQ ID 9	SSBP1	5'Fam-3'MGB labeled probe	ATGGCACAGAATATCAG
	SEQ ID 7	SSBP1	Forward primer	AGTTTACCAACTGGGTGATGTCAG
	SEQ ID 8	SSBP1	Reverse primer	TTGATATGCCACGTCTCTGAGG
26	SEQ ID 94	TNS1	5'Fam-3'MGB labeled probe	CACGGCATCCCCAAC
	SEQ ID 95	TNS1	Forward primer	AAGCCCTTGTTTCTGCACCA
	SEQ ID 96	TNS1	Reverse primer	GCCGACATCCTCCTTTAGACTC
27	SEQ ID 97	TUBA	5'Fam-3'MGB labeled probe	CGGGCTGTGTTTGTAGA
	SEQ ID 98	TUBA	Forward primer	GACTCCTTCAACACCTTCTTCAGTG
	SEQ ID 99	TUBA	Reverse primer	TGCGAACTTCATCAATGACTGTG

CLAIMS:

1. A method for predicting the recurrence of prostate cancer comprising:

(i) measuring expression levels of at least two diagnostic markers selected from among ACTG2, CALD1, CBX3, DCHS1, DKK3, DPT, FLNA, FLNC, GAS1, GSN, LIMS2, LMOD1, MT1X, MYH11, MYLK, PDLIM3, PDLIM7, RASL12, SH3BGRL, SMTN, SORBS1, SSBP1, and TNS1,

wherein each expression level is measured relative to an average level of two or more control markers; and

(ii) combining the expression level of the at least two diagnostic markers with a predictive probability based on a Kattan nomogram into a score corresponding to a probability of recurrence of prostate cancer.

2. The method of claim 1, wherein the Kattan nomogram is a composite measure based on two or more members of a group consisting of:

PSA value prior to surgery, PSA value at the time of prostatectomy, primary Gleason at surgery, secondary Gleason at surgery, prostatectomy Gleason, the year of prostatectomy, the months disease free, whether or not surgical margins were positive, whether or not cancer was found in seminal vesicles, whether or not there was extra-capsular extension, whether or not cancer was found in lymph nodes, pre-radiotherapy PSA value, radiation dose, whether surgical margins were positive or negative, whether there was seminal vesicle involvement, whether there was lymph node involvement, whether there was extra capsular involvement, whether or not neo-adjuvant hormones were prescribed, and whether or not neo-adjuvant radiation was prescribed.

3. The method of claim 1, wherein the Kattan nomogram is a composite measure based on two or more members of a group consisting of pretreatment PSA level, combined

Gleason grade, specimen Gleason sum, clinical stage, surgical margin status, prostatic capsular invasion maximum cancer length in a core, total length of cancer in the biopsy cores, percent of cores positive level, extraprostatic extension, level of extraprostatic extension, apoptotic index, percent of cancer in one or more cores, percent of high grade cancer in one or more cores, total tumor volume, zone of location of the cancer, presence of seminal vesicle invasion, type of seminal vesicle invasion, p53, Ki-67, p27, DNA ploidy status, lymph node status, and lymphovascular invasion.

4. The method of claim 1, wherein the set of markers comprises diagnostic markers MYH11, SSBP1, and DPT.

5. The method of claim 4, wherein the set of markers further comprises diagnostic markers selected from among ACTG2, CALD1, CBX3, DCHS1, DKK3, FLNA, FLNC, GAS1, GSN, LIMS2, LMOD1, MT1X, MYLK, PDLIM3, PDLIM7, RASL12, SH3BGRL, SMTN, SORBS1, and TNS1.

6. The method of claim 1, wherein the control markers are TUBA, ALAS1 and ACTG1.

7. The method of claim 1, wherein control markers are selected for steady expression levels as detected by RT-PCR.

8. A method for predicting the recurrence of prostate cancer comprising:

determining an expression level of DPT relative to a control marker;

determining expression level of at least one additional marker selected from among ACTG2, CALD1, CBX3, DCHS1, DKK3, FLNA, FLNC, GAS1, GSN, LIMS2, LMOD1, MT1X, MYH11, MYLK, PDLIM3, PDLIM7, RASL12, SH3BGRL, SMTN, SORBS1, SSBP1, and TNS1; and

transforming the expression level of DPT and the expression level of the at least one additional marker into a score corresponding to a probability of recurrence of prostate cancer.

9. A method of predicting the recurrence of prostate cancer comprising:

determining an expression level of DPT relative to a control marker;

determining expression level of at least one additional marker selected from among ACTG2, CALD1, CBX3, DCHS1, DKK3, FLNA, FLNC, GAS1, GSN, LIMS2, LMOD1, MT1X, MYH11, MYLK, PDLIM3, PDLIM7, RASL12, SH3BGRL, SMTN, SORBS1, SSBP1, and TNS1; and

combining the expression level of DPT and the expression level of the at least one additional marker with at least one additional indicator of prostate cancer recurrence to determine a composite score corresponding to a probability of recurrence of prostate cancer.

10. The method of claim 8 or 9, wherein the expression level of DPT and the expression level of the at least one additional marker is determined prior to at least one treatment selected from the group consisting of prostectomy, hormone therapy, single agent chemotherapy, two agent chemotherapy and treatment with a farnesyl transferase inhibitor.

11. A kit for use in a method of any one of claims 8-10, the kit comprising reagents and a first probe set comprising a first probe specifically recognizing DPT and a second probe specifically recognizing a second marker selected from among ACTG2, CALD1, CBX3, DCHS1, DKK3, FLNA, FLNC, GAS1, GSN, LIMS2, LMOD1, MT1X, MYH11, MYLK, PDLIM3, PDLIM7, RASL12, SH3BGRL, SMTN, SORBS1, SSBP1, and TNS1, wherein the kit comprises reagents necessary for RT-PCR.

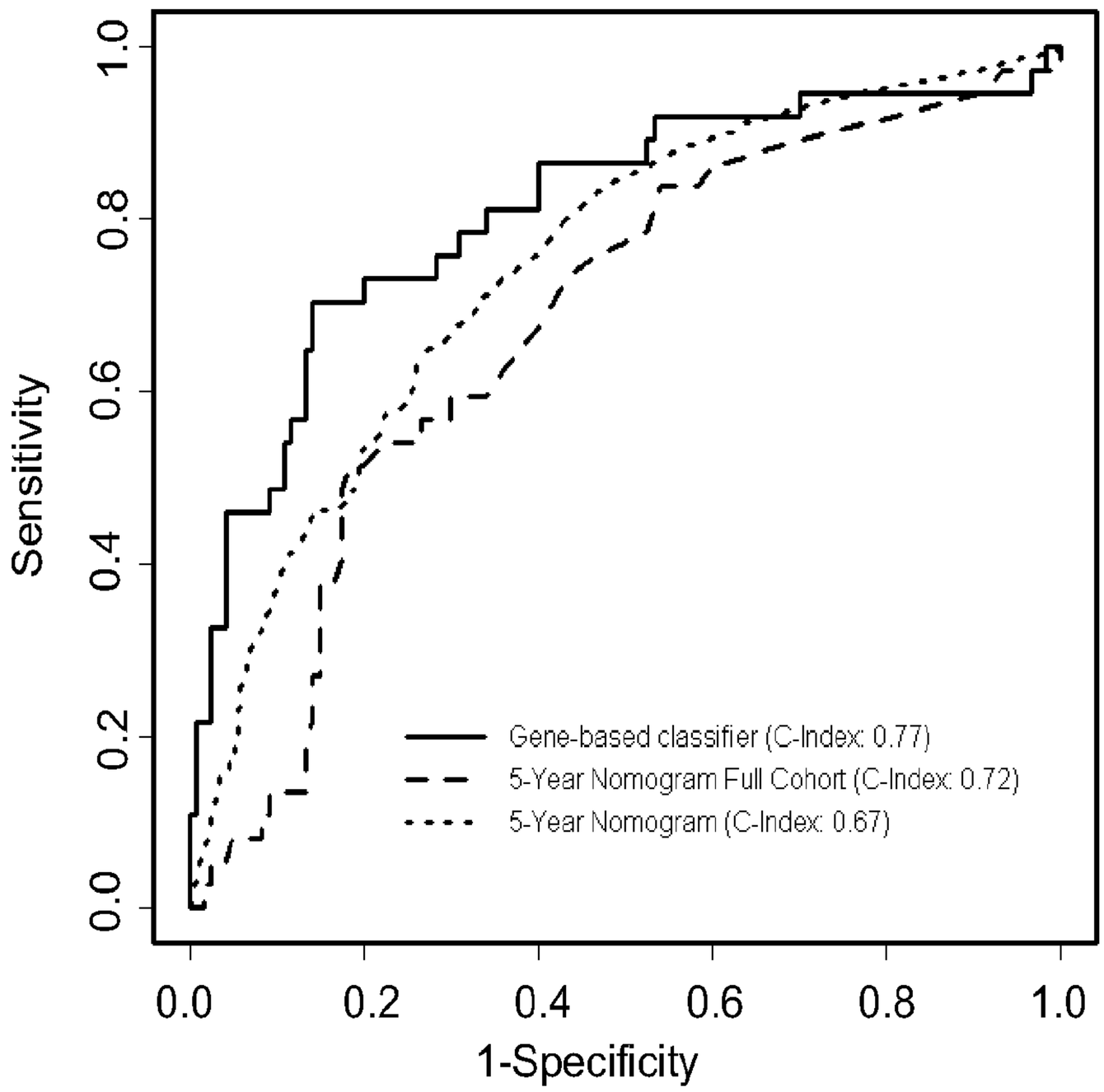
12. The kit of claim 13, further comprising a device for converting the expression level of DPT and the expression level of the second marker into an indicator of a likelihood of recurrence of prostate cancer.

13. The kit of claim 12, wherein the device is a nomogram.

14. The kit of claim 13, wherein the nomogram is implemented as a mechanical device, a graphical representation, or generated using software instructions to implement a user interface for providing a representation of the indicator of the likelihood of recurrence of prostate cancer.

Figure 1

A



B

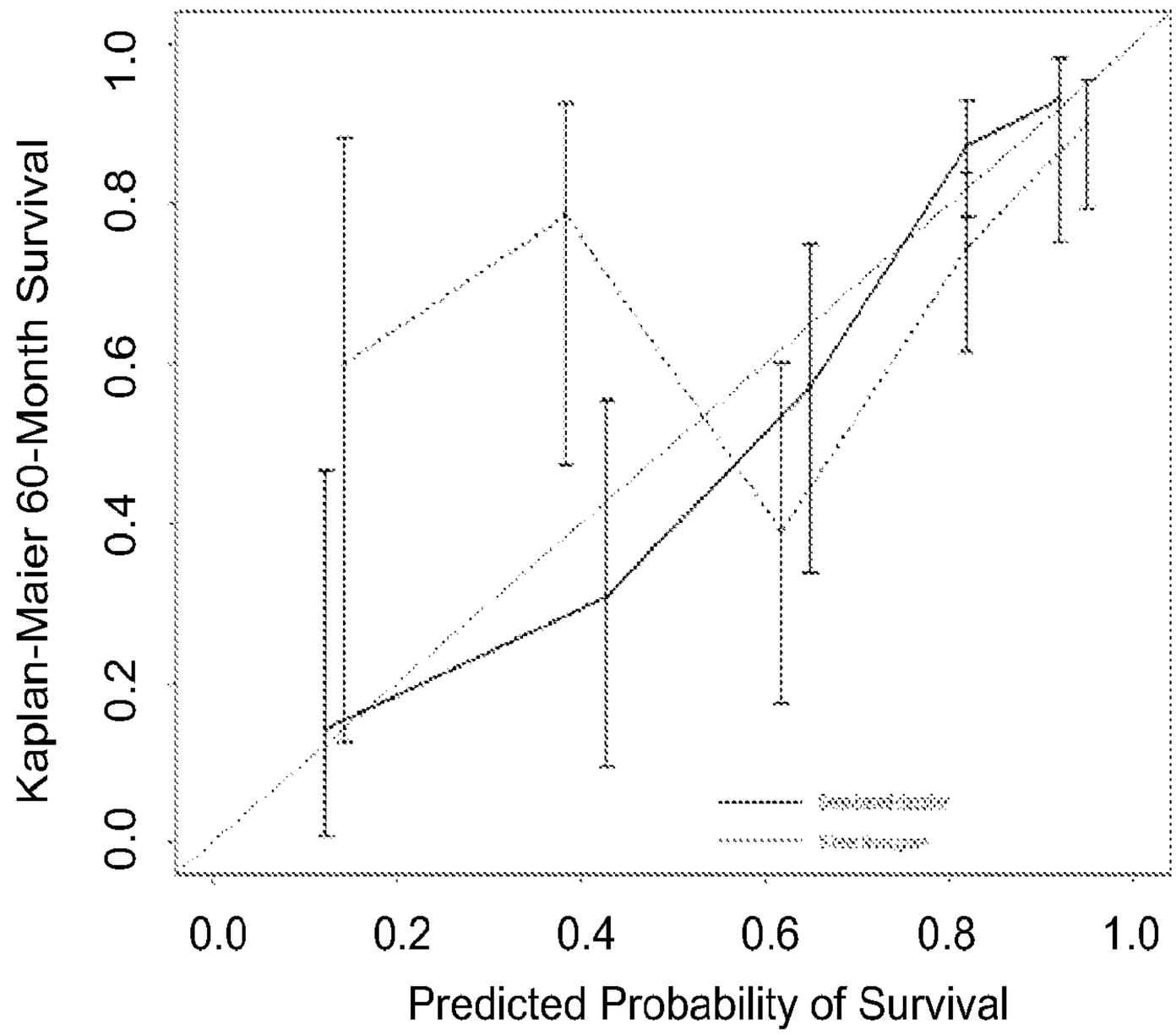


Figure 2A

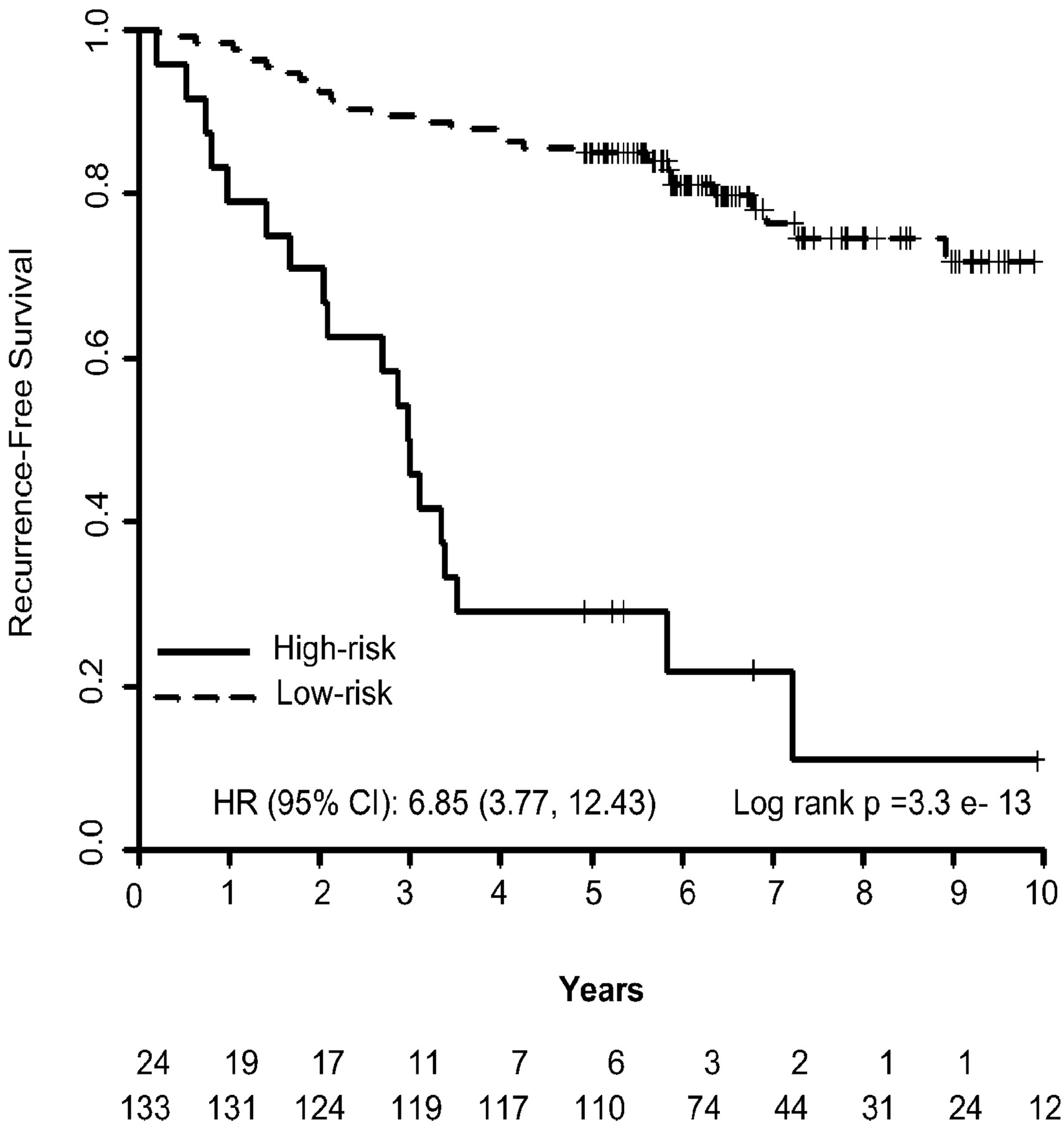


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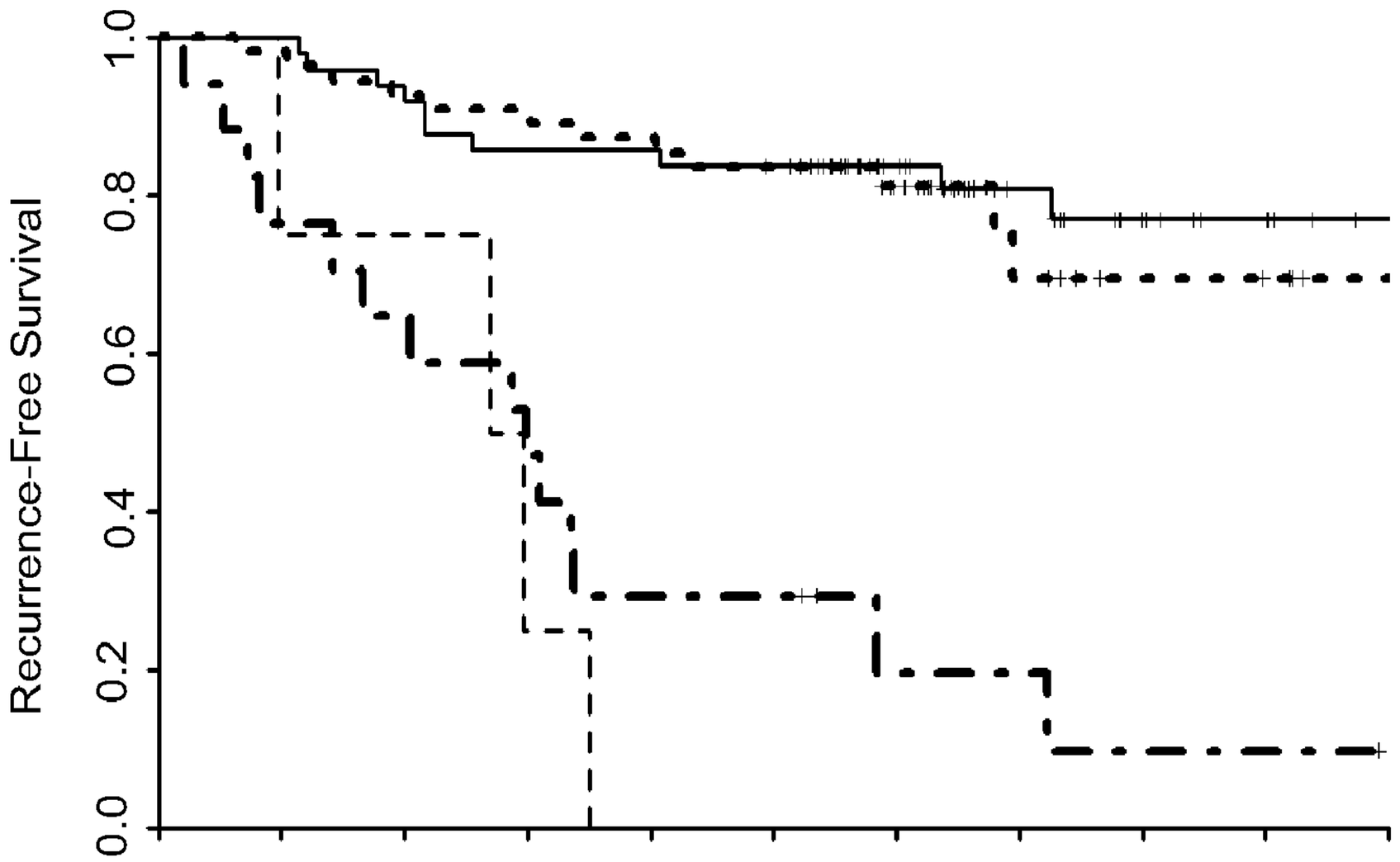
Figure 2B

GL=6 HR (95% CI): 9.44 (2.73, 32.59) Log rank p =1.5 e- 05

GL=7 HR (95% CI): 6.59 (3.01, 14.43) Log rank p =6.9 e- 08



GL=6 High-risk	4	3	3	1						
GL=6 Low-risk	49	49	46	42	42	41	32	21	13	9	4
GL=7 High-risk	17	13	11	8	5	5	2	2	1	1	
GL=7 Low-risk	55	54	51	50	48	44	28	12	8	7	4

GL=6 High-risk

GL=6 Low-risk

Gl =7 High-risk

GL =7 Low-risk

Figure 2C

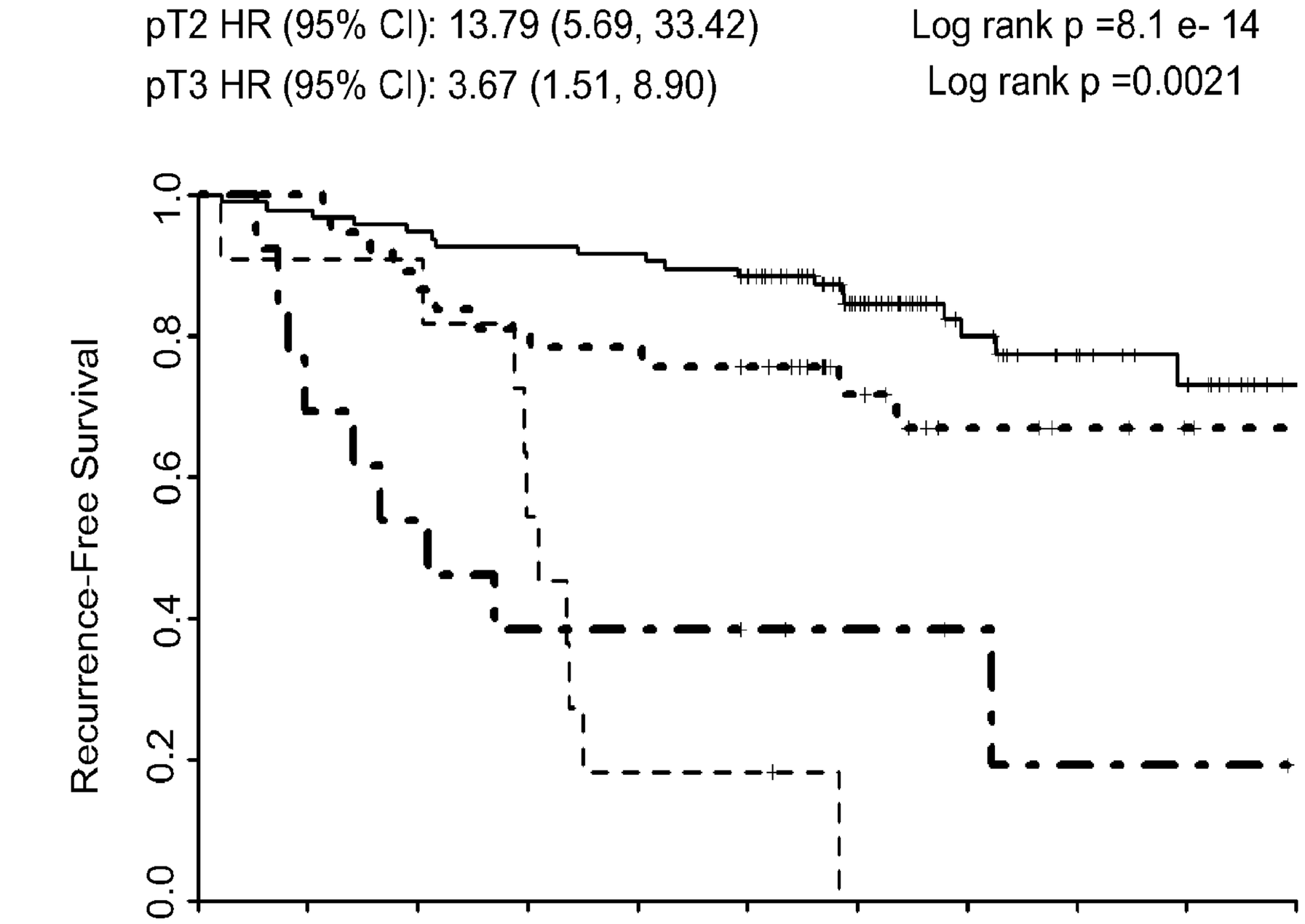

pT2 HR (95% CI): 13.79 (5.69, 33.42)
Log rank p =8.1 e- 14
pT3 HR (95% CI): 3.67 (1.51, 8.90)
Log rank p =0.0021
Recurrence-Free Survival
1.0
0.8
0.6
0.4
0.2
0.0
0 1 2 3 4 5 6 7 8 9 10
Years
pT2 High-risk 11 10 10 6 2 2
pT2 Low-risk 96 94 91 89 88 83 56 33 22 17 6
pT3 High-risk 13 9 7 5 5 4 3 2 1 1
pT3 Low-risk 37 37 33 30 29 27 18 11 9 7 6
pT2 High-risk
pT2 Low-risk
pT3 High-risk
pT3 Low-risk

Figure 2D

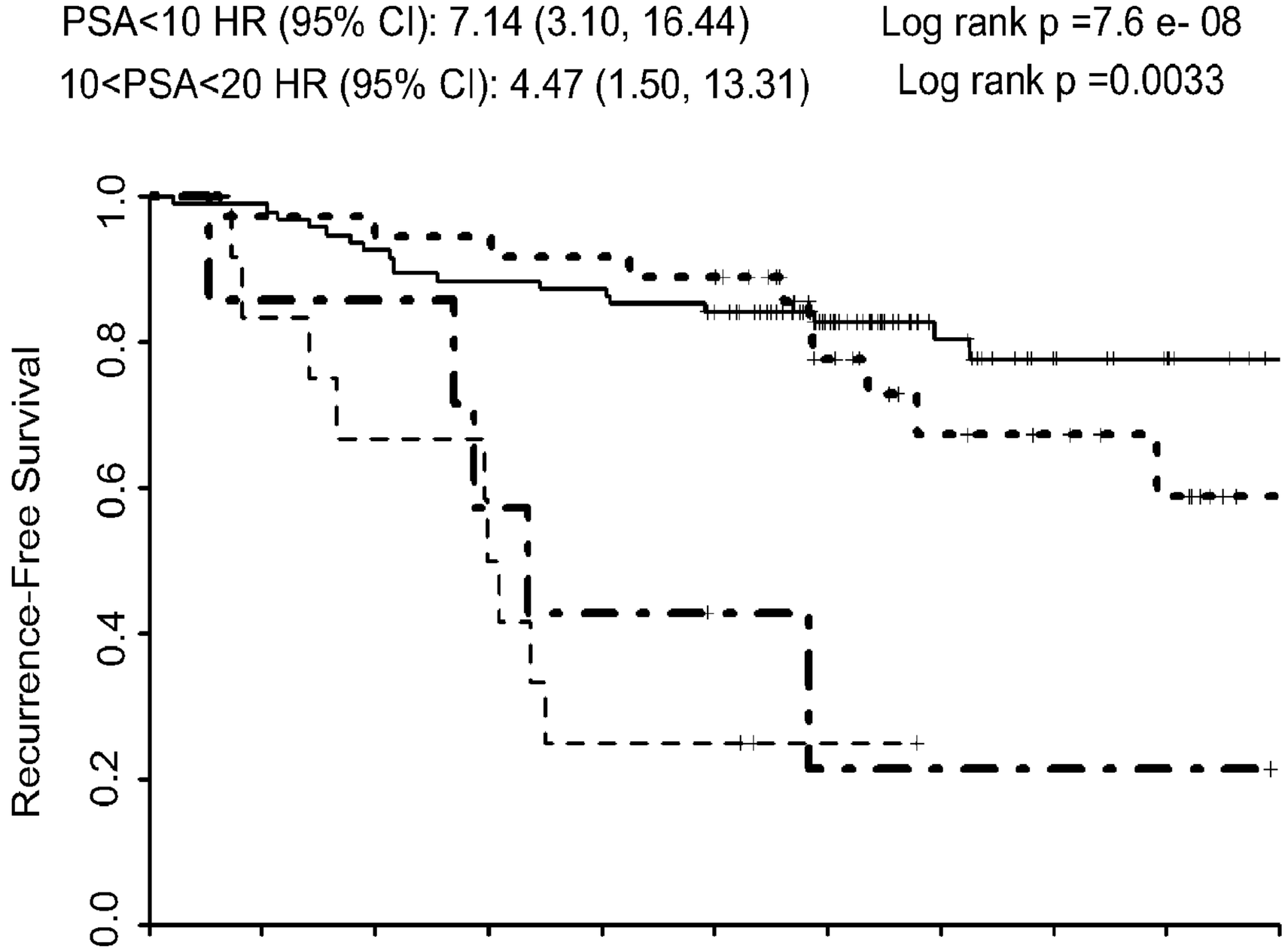
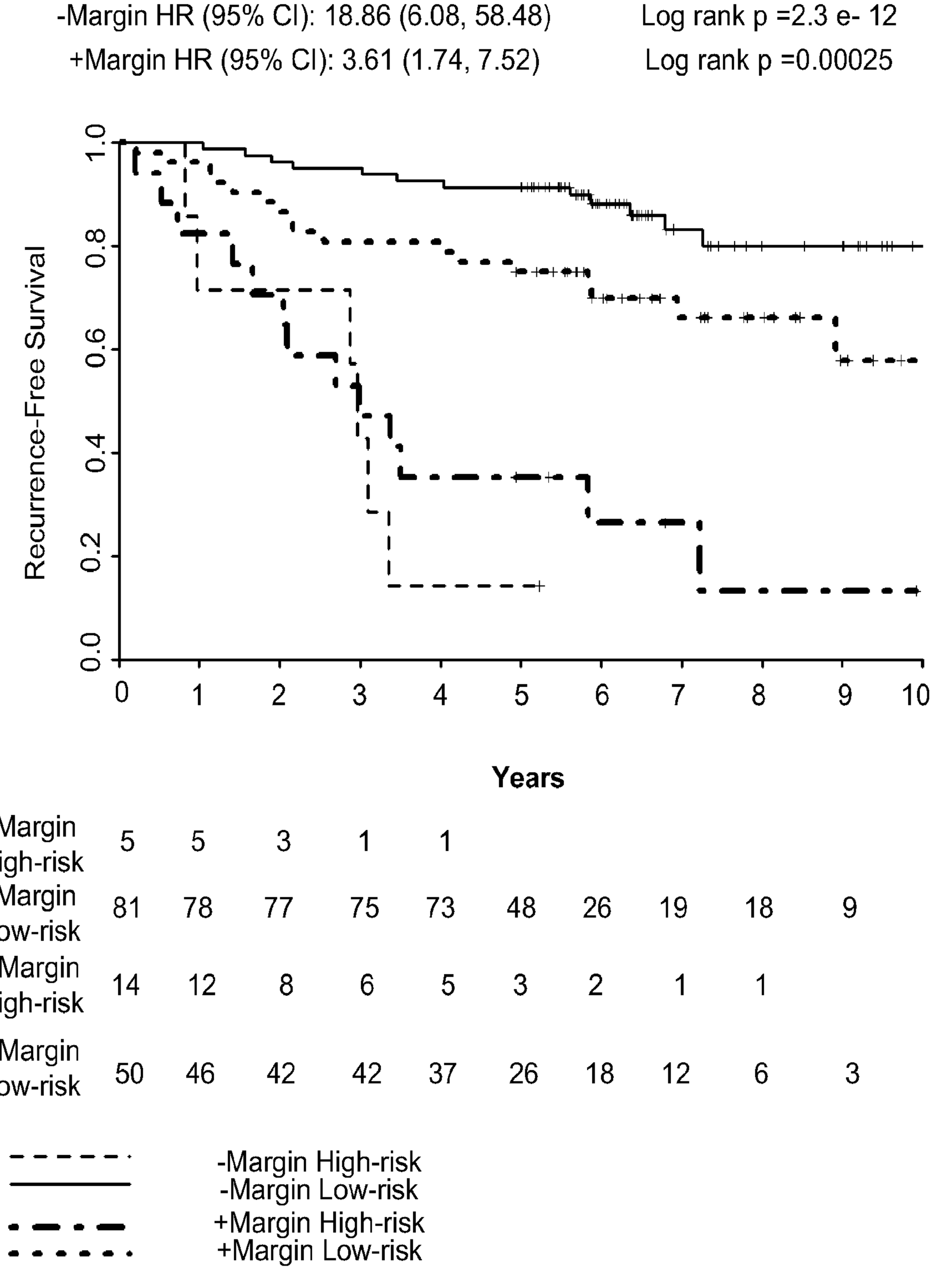

PSA<10 HR (95% CI): 7.14 (3.10, 16.44)
Log rank p =7.6 e- 08
10<PSA<20 HR (95% CI): 4.47 (1.50, 13.31)
Log rank p =0.0033
Recurrence-Free Survival
1.0
0.8
0.6
0.4
0.2
0.0
0 1 2 3 4 5 6 7 8 9 10
Years
PSA<10 High-risk 12 10 8 6 3 3 1
PSA<10 Low-risk 95 94 88 84 83 77 55 32 21 17 11
10<PSA<20 High-risk 7 6 6 4 3 2 1 1 1 1
10<PSA<20 Low-risk 36 35 35 34 33 32 19 12 10 7 1
PSA<10 High-risk
PSA<10 Low-risk
10<PSA<20High-risk
10<PSA<20Low-risk

Figure 2E


-Margin HR (95% CI): 18.86 (6.08, 58.48)
Log rank p =2.3 e- 12
+Margin HR (95% CI): 3.61 (1.74, 7.52)
Log rank p =0.00025
Recurrence-Free Survival
0.0
0.2
0.4
0.6
0.8
1.0
0 1 2 3 4 5 6 7 8 9 10
Years
-Margin High-risk 5 5 3 1 1
-Margin Low-risk 81 78 77 75 73 48 26 19 18 9
+Margin High-risk 14 12 8 6 5 3 2 1 1
+Margin Low-risk 50 46 42 42 37 26 18 12 6 3
-Margin High-risk
-Margin Low-risk
+Margin High-risk
+Margin Low-risk

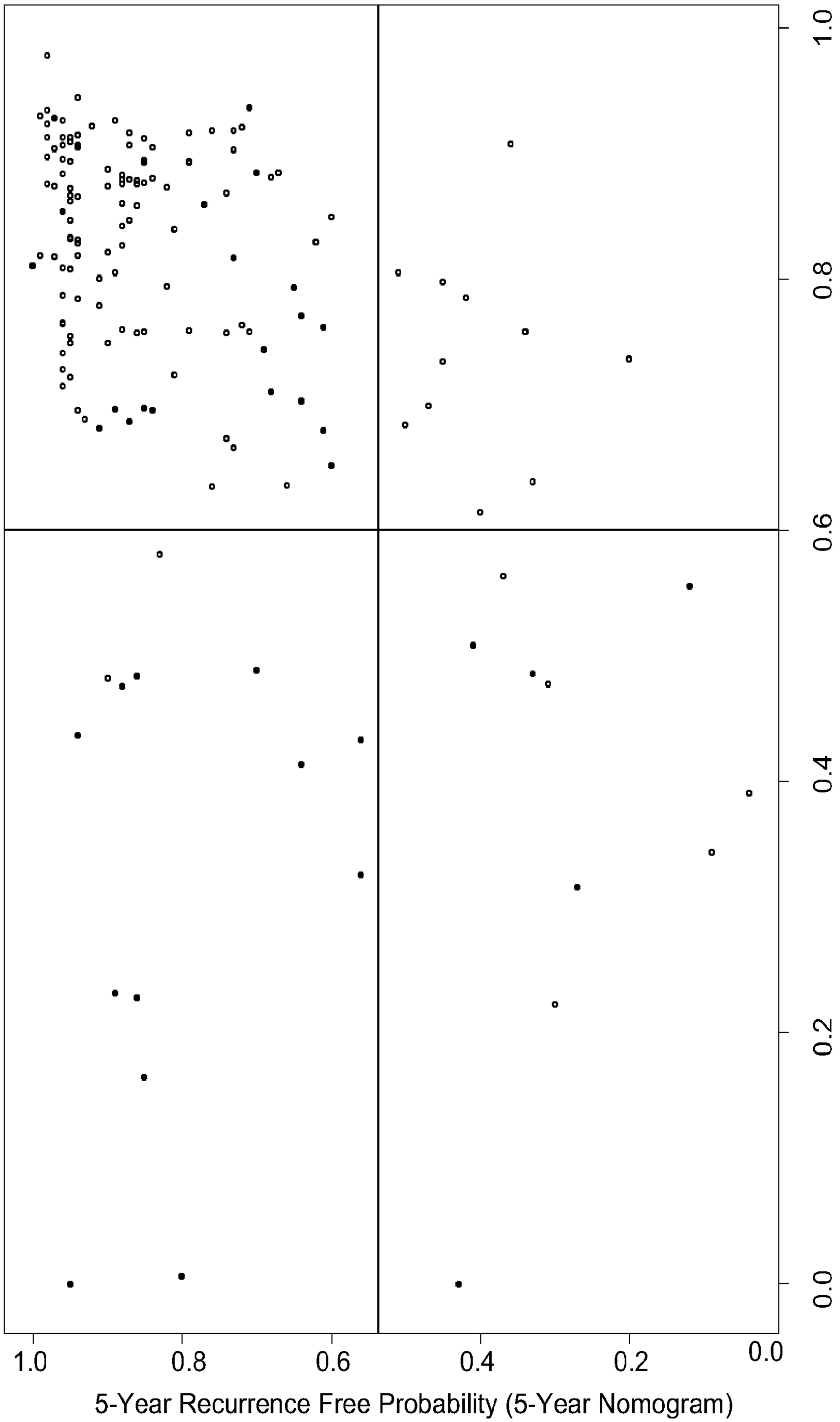

1.0
0.8
0.6
0.4
0.2
0.0
5-Year Recurrence Free Probability (Gene-Based Classifier)
1.0
0.8
0.6
0.4
0.2
0.0
5-Year Recurrence Free Probability (5-Year Nomogram)

Figure 3