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Authors

Hawley, Sarah
Fazli, Ladan
McKenney, Jesse K
et al.

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A model for the design and construction of a resource for the validation of prognostic prostate cancer biomarkers: the Canary Prostate Cancer Tissue Microarray

Sarah Hawley, M.S.[1], **Ladan Fazli, M.D.**[2], **Jesse K. McKenney, M.D.**[3], **Jeff Simko, M.D., Ph.D.**[4,5], **Dean Troyer, M.D.**[6,7], **Marlo Nicolas, M.D.**[7], **Lisa F. Newcomb, Ph.D.**[8], **Janet E. Cowan, M.A.**[4], **Luis Crouch, M.S.**[9], **Michelle Ferrari, R.N.**[10], **Javier Hernandez, M.D.**[11], **Antonio Hurtado-Coll, M.D.**[2], **Kyle Kuchinsky**[4], **Janet Liew, B.S.**[2], **Rosario Mendez-Meza**[7], **Elizabeth Smith, M.S.**[6], **Imelda Tenggara**[4], **Xiaotun Zhang, M.D.**[8], **Peter R. Carroll, M.D., M.P.H.**[5], **June M. Chan, Sc.D.**[5,13], **Martin Gleave, M.D.**[2], **Raymond Lance, M.D.**[14], **Daniel W. Lin, M.D.**[8], **Peter S. Nelson, M.D.**[12], **Ian M. Thompson, M.D.**[11], **Ziding Feng, Ph.D.**[15], **Lawrence D. True, M.D.**[16], and **James D. Brooks, M.D.**[10]

[1]Canary Foundation, Palo Alto, CA

[2]The Vancouver Prostate Centre, University of British Columbia, Vancouver, BC, Canada

[3]Department of Pathology, Cleveland Clinic, Cleveland, Ohio

[4]Department of Pathology, University of California San Francisco, San Francisco, California

[5]Department of Urology, University of California San Francisco and Helen Diller Family Comprehensive Cancer Center, San Francisco, California

[6]Department of Pathology and Department of Microbiology and Molecular Cell Biology, Eastern Virginia Medical School, Norfolk, Virginia

[7]Department of Pathology, University of Texas Health Science Center at San Antonio, San Antonio, Texas

[8]Department of Urology, University of Washington, Seattle, Washington

[9]Department of Biostatistics, University of Washington, Seattle, Washington

[10]Department of Urology, Stanford University, Stanford, California

[11]Department of Urology, University of Texas Health Science Center at San Antonio, San Antonio, Texas

[12]Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, Washington

[13]Department of Epidemiology and Biostatistics, University of California San Francisco, San Francisco, California

[14]Department of Urology, Eastern Virginia Medical School, Norfolk, Virginia

[15]Program of Biostatistics and Biomathematics, Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington

[16]Department of Pathology, University of Washington Medical Center, Seattle, Washington

Correspondence: Sarah Hawley, Canary Foundation, 1501 South California Ave, Suite 2500, Palo Alto, CA 94304; Lawrence D. True, Room BB220, Dept. of Pathology, Box 356100, 1969 NE Pacific Street, University of Washington Medical Center, Seattle, WA 98195; James D. Brooks, Stanford University School of Medicine, 300 Pasteur Drive, Room S287, Stanford, CA 94304.

Abstract

Tissue microarrays provide unique resources for rapid evaluation and validation of tissue biomarkers. The Canary Foundation Retrospective Prostate Tissue Microarray Resource used a rigorous statistical design, quota sampling, a variation of the case-cohort study, to select patients for inclusion in a multicenter, retrospective prostate cancer tissue microarray cohort. The study is designed to definitively validate tissue biomarkers of prostate cancer recurrence after radical prostatectomy. Tissue samples from over 1,000 participants treated for prostate cancer with radical prostatectomy between 1995 and 2004 were selected at six participating institutions in the United States and Canada. This design captured the heterogeneity of screening and clinical practices in the contemporary North American population. Standardized clinical data were collected in a centralized database. The project has been informative in several respects. The scale and complexity of assembling tissue microarrays (TMAs) with over 200 cases at each of six sites involved unanticipated levels of effort and time. Our statistical design promises to provide a model for outcome-based studies where tissue localization methods are applied to high-density tissue microarrays.

Keywords

Prostate Cancer; Prognosis; Tissue Microarray; quota sampling

INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer among men in the United States, with more than 200,000 new cases expected in 2012[1]. Survival following primary treatment is generally excellent, especially among men diagnosed with presumed organ-confined disease[2]. Although approximately one-third of men undergoing surgery present with clinical factors that put them at high risk of recurrence with 10-year biochemical recurrence rates as high as 30–50%, [3][4], recent screening trials have documented that many men are diagnosed with clinically indolent disease [5,6]. These statistics suggest that there are high rates of over-diagnosis and over-treatment of prostate cancer and underlie the recent recommendation by the US Preventive Task Force against routine PSA screening of men for prostate cancer [7]. Therefore, the clinical management of prostate cancer presents patients and physicians with a paradox of localized disease that is both undertreated in some and over treated in others, highlighting the critical need to identify prognostic biomarkers of prostate cancer recurrence.

The Gleason score, clinical stage, surgical margins, lymph node involvement and pre- and post-surgery PSA values, although imperfect at predicting recurrence, are widely used in the post-operative management of patients undergoing radical prostatectomy (RP). For patients who do not elect to undergo surgery or other curative therapy, digital rectal exam (DRE), repeated biopsies and PSA levels are used to monitor disease progression [8]. Several models have been constructed to predict the probability of recurrent disease both pre- and post-operatively [9,10], with the conclusion that at most 50% of variance in outcome is explained by current prognostic parameters[11]. The predictive accuracy of these models could be improved with the addition of new prognostic biomarkers [11–13].

The identification of biomarkers that associate with prostate cancer behavior will likely be derived from a deepened understanding of the underlying biology of prostate cancer aggressiveness that includes cell proliferation, survival, invasive and migratory capabilities, angiogenesis, immune system responses, and other parameters. In addition, the application and routine deployment of biomarkers requires development and standardization of molecular tools for accurate classification of the innate biological and clinical behavior.

Once identified, new molecular biomarkers associated with high risk prostate cancer need to be tested in clinical samples with detailed follow-up and established clinical endpoints. To date, most studies have focused on developing new diagnostic biomarkers to overcome the problems with PSA testing that involves addressing poor sensitivity and specificity. As such, few resources have been available for testing prognostic biomarkers, particularly for selecting patients for immediate versus deferred treatment, and monitoring disease status over time through active surveillance. Given the challenges in developing serum-based markers of prognosis, a logical first step would be to develop biomarkers that are tissue based. Biomarker testing in tissues has been expedited by the development of tissue microarrays.

Tissue microarrays (TMAs) for identifying prostate cancer biomarkers

First described in the 1980's [14,15], tissue microarrays have been used in tissue-based studies for virtually every disease, particularly human cancer. TMAs allow simultaneous evaluation of hundreds of cases on a single histologic slide and have been used for protein and nucleotide based assay systems, most commonly immunohistochemistry and in situ hybridization. Many investigators have developed prostate cancer tissue microarrays and used them in studies designed to discover and validate candidate diagnostic and prognostic biomarkers. However, despite the identification of many candidate biomarkers, very few tissue-based biomarkers have been validated across different cohorts, and fewer have been adopted for routine clinical use. The immunohistochemical markers that are routinely used in clinical work, i.e. AMACR, p63 and ERG, have been applied exclusively for diagnostic purposes, not for prognosis. To add to the confusion, multiple studies report contradictory results for a single biomarker. For example, published reports on the family of ERG fusions have described both positive and negative associations with aggressive disease [16].

There are many reasons why prognostic biomarkers have not transitioned to routine use in the clinical management of patients with prostate cancer. Many biomarkers are presented as "candidates" based on their predicting outcome in TMAs created from whatever prostate cancer samples are on hand without a probabilistic sampling scheme from a well-defined population and, most of these studies fail to test the performance of the biomarker in the context of prognostic clinical and pathological parameters currently in use, such as Gleason patterns, clinical stage or serum PSA concentrations. Furthermore, many of these TMA patient cohorts are relatively small, with limited clinical information and short or incomplete follow-up. Even when candidate biomarkers are identified in these studies, the evaluation of the markers often stops after they are identified. Lack of validation cohorts and methods of testing for clinical significance, in addition to the somewhat mundane work of testing the many candidate biomarkers in the context of clinical and pathological parameters, likely decrease the incentive to rigorously test them as prognostic markers.

Several groups have assembled TMA cohorts with hundreds of patient samples, thereby overcoming issues of inadequate power or incomplete follow-up. However, virtually all of these cohorts are derived from surgical cases from a single institution, which may limit the generalizability of the study population with regards to patient ethnicity, disease severity, type of practice. In addition, local treatment patterns and methods of follow-up also contribute to intrinsic biases of single-institution patient cohorts. Additionally, many of these larger cohorts have significant patient heterogeneity engendered by PSA screening procedures. PSA screening has resulted in a change in the spectrum of prostate cancers in the US population, with migration over time to lower tumor stage and tumor volume. In the Prostate, Lung, Colorectal and Ovarian screening trial, Gleason grades shifted significantly to lower grades in patients detected in the first round of screening compared to those detected in subsequent rounds [17]. Many TMA cohorts include a mixture of old and

contemporary patient samples that add heterogeneity to the population but might not be relevant to current sets of patients identified by intense PSA screening.

STUDY DETAILS

Rationale and design of a multi-institutional TMA platform

The Canary Foundation Retrospective Prostate Tissue Microarray Resource (CFRPTMR) is a multicenter, retrospective prostate cancer tissue microarray study undertaken as a collaborative effort between 6 academic medical centers - Stanford University, University of California, San Francisco, University of British Columbia, University of Washington, University of Texas Health Science Center at San Antonio and Eastern Virginia Medical School. The study is supported by the Canary Foundation, Palo Alto, CA. The primary objective of the study is to *validate* biomarkers that have been reported to predict recurrent prostate cancer at the time of radical prostatectomy (RP). The secondary objective of the study is to *discover* candidate biomarkers for the prediction of non-recurrent disease. The primary study endpoints are time to recurrence and five year recurrence free survival.

The discovery and validation of clinical biomarkers in many ways parallel the steps necessary for drug development. In addition to identifying a target biomarker and developing a clinically certifiable means for measuring the biomarker, the biomarker must be tested and validated on a well-defined patient population and address a relevant clinical question. Since many tissue-based biomarker candidates have been identified and standard means of measuring the markers (e.g. immunohistochemistry) are widely used in clinical practice, we surmised that the bottlenecks to biomarker development primarily lie in validation. To address the challenges of biomarker validation, we assembled a team of pathologists, clinicians, statisticians and cancer researchers and spent two years designing and creating a tissue microarray resource for validating biomarkers of prostate cancer prognosis. As the study design emerged, it became clear that the study would follow many of the principles of a prospective clinical trial in a retrospective setting. Implementing this rigorous design involved challenges that were not anticipated in the initial study planning. Although several of the challenges were specific to prostate cancer, the resulting design features are generally applicable to most tissue-based disease studies.

We designed a common TMA platform across multiple institutions to avoid the single-institution bias. We chose to test prognostic markers in prostate tissues from a radical prostatectomy cohort. This cohort was chosen because the clinical and pathological features of the cancers could be sampled robustly (e.g. cancer grade and stage), abundant tissue was available for TMA construction, and patient outcomes were well documented. Since data suggest that some contemporary patients are over-treated [18–20], the study was designed to distinguish between indolent and aggressive disease in low and intermediate risk patients.

The clinical need to distinguish indolent from aggressive disease in men undergoing prostatectomy drove the definition of study endpoint. We selected a study outcome that captured aggressiveness and clearly defined how this outcome would be measured. The gold standard for aggressive disease is recurrent or metastatic prostate cancer. However, metastatic disease typically manifests up to 10 years after initial prostate cancer treatment [4] leading to concerns about insufficient follow-up time and spectrum bias. Prostate cancer progression after surgery is typically monitored using serum PSA concentrations as a surrogate for local recurrence or metastasis. Biochemical recurrence may identify a group of patients who are at significantly higher risk for the development of metastases and prostate cancer mortality [21]. Thus, we decided to include PSA-recurrence within 5 years of RP as a study outcome in addition to secondary/salvage therapy and clinical evidence of metastasis.

Almost all biomarker candidate studies are retrospective case-control studies and thus prone to spectrum bias in which the study sample is not representative of the clinically relevant population. For retrospective case-control studies, the cases included in the study tend to have more aggressive disease and better follow-up, both in quality of data collection and length of follow-up. Similarly, the controls included in a retrospective case-control study often represent the healthiest patients with the best follow-up. For example, metastatic prostate cancer frequently manifests 10 or more years after initial treatment for prostate cancer. The natural impulse in selecting non-recurrent patients (controls) is to limit selection to non-recurrent patients with at least 10 years of follow-up, potentially leading to spectrum bias. To help reduce this bias, a small number of censored patients (i.e. patients whose recurrent status at 5 years post-RP is unknown) are included in the study design as well as a small number of patients who experience recurrence more than 5 years after RP. To eliminate institutional selection biases, TMAs were constructed at six institutions with diverse patient populations and practice patterns. A collaboration agreement, including material transfer agreements, signed by all participating sites, allows for transfer of TMA sections among participating sites.

To accurately measure the study outcome of aggressive disease and ensure that patients met the eligibility criteria, detailed follow-up data on PSA and other clinical characteristics were required. While some sites maintained an electronic database of patient information associated with stored prostatectomy samples, others did not. These sites extracted the necessary information from medical records for each patient, a laborious and time-consuming process that shaped the sampling plan for the study. Ideally, a study cohort is drawn randomly from all eligible patients in the target population, in this case, all men undergoing prostatectomy after 1995 at the participating sites. A starting date of 1995 was selected because much of the stage shift caused by PSA screening of the US population occurred prior to that year [22].

The study used a quota-sampling plan [23](see Supplementary Materials). A random list of the entire RP cohort at each site was generated and recurrent and non-recurrent cases were identified. Participants were then chosen by moving sequentially down the list, extracting information from medical records if needed, and confirming the eligibility of each patient until the targeted number of participants in the recurrent and non-recurrent categories was obtained. This approach minimized medical records extraction since selection only continued until the target number of eligible patients was identified.

One unanticipated challenge was the time and effort required to retrieve tissue blocks that had adequate material for the TMAs after patient selection was complete. At some sites, tissue blocks for selected patients had been either consumed for other studies or were missing entirely. In some cases the growth pattern of the cancer was so serpiginous that no more than a single core could be obtained of the cancer, instead of the three cores on which the TMA design was based. After consideration of the study design and discussion, we decided that in such cases one core sufficed so that such cancers were not underrepresented in the TMAs. At several sites, substantial effort was needed to locate the missing tissues, which were often scattered in several labs where the tissues had been used for other research projects.

Patient and sample selection

The study includes tissue derived from radical prostatectomy surgical specimens. The study included samples from men with a) recurrent prostate cancer, b) non-recurrent prostate cancer and c) unknown outcome due to inadequate follow-up time (i.e. censoring). Recurrent prostate cancer is defined by 1) a single serum prostate-specific antigen (PSA) level greater than 0.2 ng/mL more than 8 weeks after RP and/or 2) receipt of salvage or

secondary therapy after RP and/or 3) clinical or radiological evidence of metastatic disease after RP. Although lower thresholds for biochemical recurrence have been proposed [24], the lower bound of sensitivity of PSA testing at some sites during the study period was limited to 0.2 ng/mL. Defining biochemical recurrence at a lower PSA value would have resulted in inconsistent application of the definition. Non-recurrent prostate cancer is defined as disease with none of the indicators of recurrence for at least five years after RP. Participants with no evidence of recurrent prostate cancer but less than five years of follow-up after RP (i.e. censored) were also eligible for the study. Inclusion and exclusion criteria and definitions of recurrent and non-recurrent disease are given in Table 1. The full study protocol is available from the authors upon request.

Data Management

The participating sites transmitted de-identified patient data for all RP patients undergoing surgery during the study period to the lead statistician in the study (ZF). The study statisticians mapped the submitted data to a set of standardized clinical variables creating a secure, centralized, database of clinical and pathological information. The statistical core checked the eligibility of each participant and returned a randomized participant list to the sites for participant selection via quota-sampling, as described below and in the Supplemental Materials.. Common data elements obtained from each institution are available on request.

Participants in the centralized database were only identified by study ID, ensuring patient confidentiality. Databases linking study IDs to patient identifying information are maintained in a locked area at each study site.

TMA Construction

The TMAs consist of formalin-fixed, paraffin embedded tissue. Each site built a set of five TMAs, in duplicate, each block containing tissue from 42 participants and 8 common control tissues (colon, tonsil, kidney, healthy prostate and liver) using an 11×16 layout (See Supplementary Material AA). For each control tissue, the tissue blocks were obtained from the same patient and distributed to the sites. Use of a common control allows for comparison of assay quality across sites.

A one mm diameter needle was used to remove tissue cores from each donor tissue block. For each case, three cores of cancer tissue were obtained from the highest grade cancer in the dominant tumor. These cancer cores generally include regions of non-neoplastic glands. In addition, one core of histologically benign prostate glandular tissue was obtained from the peripheral zone of each case altogether yielding a total of four cores per case represented on the TMA. The cores from a single participant were grouped together on the TMA. Recurrent and non-recurrent participants were randomly distributed across the TMAs. The common control tissues were grouped together providing a visual check for slide orientation.

In addition to the cores extracted for the duplicate TMAs, three cores of cancer tissue were obtained from the highest grade cancer in each case and reserved for DNA or other nucleic acid biomarker discovery or validation studies. The standard operating procedures detailing TMA construction are available from the authors on request.

TMA Distribution

A collaboration agreement, including material transfer agreements, executed at all participating sites, allows for transfer of TMA sections among participating sites. The TMA resource is also available to outside investigators. Applications to use the TMA resource are considered by the Review Committee, consisting of investigators from each participating

site. Applications are available through the Canary Foundation (www.canaryfoundation.org).

Whenever possible, digital images of stained TMA sections are uploaded and stored in a password protected web-accessible database that allows all sites to access and evaluate the images remotely. Staining is evaluated by study pathologists following standardized procedures. Evaluation procedures vary depending on the staining qualities of the particular biomarker under evaluation.

Statistical Considerations

Avoiding over-treatment of men with non-recurrent disease requires a highly specific biomarker. Hence, to validate a candidate biomarker of recurrence, we estimate the sensitivity at the threshold level associated with 98% specificity by constructing a time-dependent ROC curve for recurrence within 5 years of RP [25]. Time-dependent ROC curves offer several advantages as a tool for validating biomarkers. First, ROC curves in general are not dependent on disease prevalence. Thus, sensitivity and specificity of a biomarker can be estimated from a case-cohort study. Second, time-dependent ROC curves incorporate information from censored patients, reducing the potential bias from including only non-recurrent patients with more than 5 years of follow up.

With specificity set at 98%, we assume a biomarker must demonstrate 30% sensitivity to be clinically useful in identifying recurrent disease. This is approximately double the 15% sensitivity of Gleason score which remains the most powerful clinically applicable single variable predicting outcome in prostate cancer. The sample size needed to achieve 90% power to detect sensitivity of 30% or greater at 98% specificity is 393 recurrent patients and 393 non-recurrent patients (see Supplemental Materials for detailed calculation). The participating sites each contributed approximately equal numbers of recurrent and non-recurrent participants to the study, and the number of participants was distributed nearly evenly across the study sites. The total sample size of 1176 ensures adequate power and accounts for the 15–30% of cores that typically drop out when a TMA is sectioned [26].

Patients were selected using quota-sampling, a variation of the traditional case-cohort design described earlier. When selecting cases for study inclusion, the sampling probability for non-recurrent Gleason score 8–10 patients and recurrent Gleason 6 patients is doubled to oversample these groups of patients, who are of special interest. Table 2 details the study participant characteristics.

Project timetable

Compared to a TMA study using a convenience sample of available tissues from a single institution, construction of the Canary Foundation Retrospective Prostate Tissue Microarray Resource required a considerable increase in effort. Creating a multi-center resource coupled with a rigorous statistical design was a major effort and the time from finalizing the study protocol to construction was several years. Steps to construction included selection and standardization of clinical and pathological Common Data Elements, design and testing of the TMA layout, completion of a multi-site Material Transfer Agreement, case identification and selection of blocks based on review of sections. In particular, obtaining, reviewing, and selecting slides and blocks from potentially eligible cases required substantial effort at each site. Many slides and blocks were either not available or had been consumed by other studies. To confirm study eligibility for over 200 cases required that a pathologist at each institution review slides from at least 220 cases. This process involved annotating up to 5,500 slides (25 slides from each of 220 cases). An additional staff person was hired at several institutions to obtain slides for the pathologist to review.

DISCUSSION

The Canary Foundation Retrospective Prostate Tissue Microarray Resource is a carefully constructed TMA cohort designed to both definitively validate candidate biomarkers of aggressive disease at the time of radical prostatectomy and to discover new biomarkers for non-recurrent disease that can be used to help select patients for active surveillance. Our intent is to test these candidate biomarkers in an established a prospective, multi-institutional cohort, the Canary Prostate Active Surveillance Study (PASS), to determine whether these prognostic biomarkers can be used for selection of men at low risk for progression on active surveillance [8]. By carrying out both discovery and validation studies in tandem, we attempt to address the critical question of which patients with localized prostate cancer can be safely watched and which patients require immediate therapy.

Unlike other large prostate TMA cohorts, patients have been selected according to a strict protocol, using design features similar to a clinical trial. This design offers significant advantages in decreasing potential biases inherent in many tissue microarray studies. First, by selecting patients randomly from institutional radical prostatectomy cohorts, we minimize spectrum bias. Second, by distributing patients across institutions we make our results more generalizable by decreasing the influences engendered by local patient selection biases, differences in treatment and variations in follow-up and endpoint assessments. Third, the prospective involvement of statistical experts allowed careful definition of study endpoints and power calculations that will render positive and negative findings of tested biomarkers clinically meaningful. Our objective was to design a study in which tissue based biomarkers could be assessed using methods that were up to standards necessary for regulatory approval for use in the clinic. Given these strengths, the statistical design of this study may serve as a model for future outcomes-based studies in other diseases that employ tissue-based biomarkers. In addition, our tissue microarray is a resource available to the cancer research community for the evaluation of prognostic biomarkers with sufficient preliminary data to justify testing.

Constructing tissue microarrays using the approach and standards we have detailed entails challenges and costs. The time from initially planning to final construction and use of the microarrays was much longer than anticipated. A significant portion of that time was spent in the design of the study. However, in the long-term, we anticipate that investigators using and adapting our study design can save significant time and the output in terms of confidence in the performance of a given biomarker is enhanced. Even with our methods, study planning requires significant input from a dedicated statistician, as well as assessment of data quality from sites, and direct participation in quota sampling. In addition, use of this study design relies on the availability (or creation) of patient databases at participating institutions. These data must be transferred to the statistician(s) at a central data site in a secure and blinded fashion which requires a database manager at each site. There are also significant challenges in the construction of the TMAs. Obtaining appropriate blocks on specific selected cases from pathology archives can be rate limiting. Furthermore, as in all tissue microarray studies, our study required significant time and commitment on the part of the study pathologists, who had to review all cases, select the dominant tumor, mark the blocks for core harvesting, supervise array construction and perform quality control on the final microarrays. While these challenges can be substantial, we have demonstrated that they are surmountable.

The study design imposes certain limitations. Definitive validation of biomarkers of non-recurrent disease requires biopsy tissue taken at the time of diagnosis, i.e. when a patient would be evaluated for entry into an active surveillance program. Biopsies produce a much smaller volume of cancer for biomarker discovery and validation. This study will select a

small set of candidates for further validation with those precious biopsy samples. Other limitations include effects associated with sampling tissue blocks to construct TMAs. By using cores to represent the entire tumor, we may miss an 'index' lesion that is actually responsible for disease progression.

As we embark on assessment of prognostic biomarkers in this cohort, we will continue to test and refine the use of this resource. We anticipate that the quality of the resource will be sufficient to allow definitive testing of tissue biomarkers that they may be translated to clinical use. We encourage use of this resource by the prostate cancer research community for evaluation of mature prognostic biomarkers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Study inclusion/exclusion criteria.

Inclusion Criteria

- Radical prostatectomy surgery occurred between January 1, 1995 and September 15, 2004
- Required clinical data are available
- Required amount of tissue is available

Exclusion criteria

- PSA levels less than 0.2 ng/mL for 6 months after RP in conjunction with adjuvant therapy of any type
- Neoadjuvant hormone therapy

Recurrent prostate cancer

- Disease with evidence of recurrence at any time after radical prostatectomy as measured by:

  1) a single serum prostate-specific antigen (PSA) level greater than 0.2 ng/mL more than 8 weeks after RP

  and/or 2) receipt of salvage therapy after RP

  and/or 3) clinical or radiological evidence of metastatic disease.

Non-recurrent prostate cancer

- Disease with no evidence of recurrence for at least 5 years after RP as measured by:

  1) serum PSA level less than 0.2 ng/mL for the entire follow-up period

  and 2) no receipt of salvage therapy

  and 3) no clinical or radiological evidence of metastatic disease.

Definitions

- Neoadjuvant hormone therapy => hormone therapy of any type received prior to RP.
- Adjuvant therapy => radiation, chemotherapy or hormone treatment received less than 6 months after RP.
- Salvage therapy => radiation, chemotherapy, hormone treatment or surgery received more than 6 months after RP.

RP=radical prostatectomy

Table 2

Summary of study participants by recurrence status, follow-up time after radical prostatectomy, Gleason score and site.

	Stanford	UCSF	UW	UBC	UT	EVMS	Total
Recurrent Patients	88 (18%)	101 (20%)	102 (21%)	20 (4%)	81 (16%)	103 (21%)	495
<5 yrs. FU post-RP	72	91	92	14	71	92	432
<=6	19	25	19	1	14	48	126
3+4	27	52	27	7	24	33	170
4+3	35	10	35	3	6	6	95
8–10	11	4	11	1	25	5	57
UNK	0	0	0	2	2	0	4
>5 yrs. FU post-RP	16	10	10	6	10	11	63
<=6	2	3	3	3	6	3	20
3+4	9	5	5	1	2	5	27
4+3	4	3	1	1	1	1	11
8–10	1	0	1	1	1	2	6
	Stanford	**UCSF**	**UW**	**UBC**	**UT**	**EVMS**	**Total**
Non-recurrent Patients	84 (14%)	98 (16%)	106 (17%)	100 (16%)	127 (20%)	106 (17%)	621
<5 yrs. FU post-RP	13	9	10	19	24	12	87
<=6	1	4	4	7	15	11	42
3+4	9	4	2	11	6	1	33
4+3	2	1	1	1	2	0	7
8–10	1	0	3	0	1	0	5
>5 yrs. FU post-RP	71	89	96	81	103	94	534
<=6	22	40	52	50	55	59	278
3+4	37	41	37	22	30	32	199
4+3	9	7	6	5	6	3	36
8–10	3	1	1	4	12	0	21

RP=radical prostatectomy

FU=follow-up