Validation and Derivation of miRNA-Based Germline Signatures Predicting Radiation Toxicity in Prostate Cancer

Amar U. Kishan[1], Kristen McGreevy[2], Luca Valle[1,3], Michael Steinberg[1], Beth Neilsen[1], Maria Casado[1], Minsong Cao[1], Donatello Telesca[2], and Joanne B. Weidhaas[1]

ABSTRACT

Purpose: Although radiotherapy (RT) is one of the primary treatment modalities used in the treatment of cancer, patients often experience toxicity during or after treatment. RT-induced genitourinary (GU) toxicity is a significant survivorship challenge for patients with prostate cancer, but identifying those at risk has been challenging. Herein, we attempt (i) to validate a previously identified biomarker of late RT-induced GU toxicity, PROSTOX, consisting primarily of miRNA-based germline biomarkers (mirSNPs), and (ii) investigate the possibility of temporally and genetically defining other forms of RT-associated GU toxicity.

Experimental Design: We included 148 patients enrolled in Magnetic Resonance Imaging-Guided Stereotactic Body Radiotherapy for Prostate Cancer (MIRAGE; NCT 04384770), a trial comparing MRI- versus CT-guided prostate stereotactic body RT. Linear regression was used to evaluate the association between PROSTOX score and late GU grade toxicity. Machine learning approaches were used to develop predictive models for acute toxicity and chronic GU toxicity, and the accuracy of all models was assessed using AUC metrics. A comparative Gene Ontology analysis was performed.

Results: PROSTOX accurately predicts late GU toxicity, achieving an AUC of 0.76, and demonstrates strong correlation with GU toxicity grade (p-1.2E−9). mirSNP-based signatures can distinguish acute RT-associated GU toxicity and chronic RT-associated GU toxicity (AUCs of 0.770 and 0.763, respectively). Finally, Gene Ontology analysis identifies unique pathways involved in each form of GU toxicity: acute, chronic, and late.

Conclusions: These findings provide strong evidence for the continued application of mirSNPs to predict toxicity to RT and act as a path for the continued personalization of RT with improved patient outcomes.

Introduction

Radiotherapy (RT) is a treatment that has been used for over a century (1) in the management of all cancers, including prostate cancer, and has been dramatically transformed and improved through the application of advanced technologies (2). Along with an appreciation of the high sensitivity of prostate cancer to larger RT doses per fraction, these technological advances have led to the introduction of stereotactic body RT (SBRT), which allows the delivery of radiation in as few as five fractions instead of the prior standard course of radiation of 8 to 9 weeks [referred to as conventionally fractionated RT (CFRT)]. SBRT is now considered a standard-of-care option for localized prostate cancer with noninferior outcomes (3–5).

Unfortunately, RT-associated long-lasting toxicity continues to be a problem for patients, including even those treated with the most advanced treatment delivery methods (6, 7). The significance of long-lasting toxicity is especially problematic for patients with prostate cancer, given their excellent prognosis and the high incidence of the disease (8). The most frequent toxicity experienced by patients with prostate cancer is genitourinary (GU) toxicity, which includes symptoms such as urinary frequency, urgency, retention, dysuria, hematuria, and a weak urinary stream (9). Currently, two "types" of RT-associated toxicity are clinically reported, acute toxicity, toxicity that occurs during or within 3 months after treatment, and late toxicity, toxicity that presents 6 months or later after treatment. However, there is an additional form of RT-induced toxicity that, although not separately clinically reported, is recognized, which is referred to as consequential or chronic toxicity (3, 10). Chronic toxicity is experienced when acute toxicity does not resolve and may explain the previously identified association between acute and late toxicity (10). Currently, although it is unknown if patients who develop chronic toxicity differ from those who develop acute-only or late toxicity, these patients are counted in both groups owing to the early presentation and slow resolution of this form of toxicity.

Although the original studies of RT-induced toxicity centered around countermeasures to manage risks of lethal large whole-body doses of RT (11), current work has focused on RT-induced toxicity experienced by patients treated with curative intent RT. Some toxicity can be attributed to clinical factors (12) and dose to organs at risk, such as the bladder in the case of prostate cancer (13, 14). However, even as more precise targeting and improved dosimetry to organs at risk is realized through advancements in technology, toxicity remains, suggesting that some degree of toxicity is due to inherent patient-specific radiosensitivity. Attempts to identify patient-specific RT toxicity biomarkers have interrogated the germline DNA (15–18). Most recently, germline variants

[1]Department of Radiation Oncology, University of California Los Angeles, Los Angeles, California. [2]Department of Biostatistics, University of California Los Angeles, Los Angeles, California. [3]Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, California.

Corresponding Author: Joanne B. Weidhaas, Department of Radiation Oncology, University of California Los Angeles, 200 Medical Plaza, Suite B265, Los Angeles, CA 90095. E-mail: jweidhaas@mednet.ucla.edu

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Translational Relevance

Toxicity following radiotherapy (RT) remains a major consideration for patients with cancer and physicians contemplating choice of treatment. In a previous study, we identified a biomarker, PROSTOX, based primarily on germline variants disrupting miRNAs and their regulatory regions (referred to as mirSNPs), which dichotomized patients with prostate cancer into low versus high risk of stereotactic body RT–induced late grade ≥2 genitourinary toxicity, with those at high risk having an approximate 12-fold increased risk. In this study, we prospectively validate PROSTOX and show that clinical and dosimetric factors do not enhance this genetic biomarker. Additionally, we demonstrate the clear existence of three separate temporal RT-induced genitourinary toxicity profiles—acute only, chronic, and late—which are also defined based on mirSNP-derived signatures. These results support that mirSNP-based biomarkers can enable appropriate counseling of patients about different types of toxicity risks prior to RT, truly personalizing radiation by determining the safest treatment choice for each patient.

based on disruption of miRNAs and their regulatory regions (referred to as mirSNPs) have been shown to be powerful predictors of both response and toxicity to RT (19–21). mirSNPs were recently applied to patients with prostate cancer to identify those at increased risk of significant late grade ≥2 GU toxicity after SBRT versus CFRT (22, 23). These studies found distinct genetic signatures predicting significant late GU toxicity for patients treated with SBRT versus those treated with CFRT (22), findings supported by prior work showing unique biological and genetic responses based of radiation fraction sizes (24). These original studies led to the development of the PROSTOX biomarker, which dichotomizes patients into those at low risk of SBRT-induced late grade ≥2 GU toxicity versus those at high risk, with those at high risk having an approximate 12-fold increased risk (22).

Here, our purpose was to independently validate PROSTOX as a predictor of SBRT-induced late significant GU toxicity in a prospective technologically driven trial, enabling consideration of carefully detailed patients' clinical and dosimetric characteristics as well as the evaluation of PROSTOX with GU toxicity grade. We also investigated the possibility of temporally and genetically defining chronic GU toxicity as a separate entity from acute-only and late toxicity using mirSNPs in conjunction with clinical and dosimetric factors.

Materials and Methods

MIRAGE

The phase III Magnetic Resonance Imaging-Guided Stereotactic Body Radiotherapy for Prostate Cancer (MIRAGE; NCT 04384770) trial evaluated whether aggressive planning target volume margin reduction enabled by MRI-guided prostate SBRT would reduce toxicity when compared with standard-of-care CT-guided prostate SBRT (6). All patients received 40 Gy in five fractions prescribed to the prostate and proximal seminal vesicles, with either a 2 mm (MRI arm) or 4 mm (CT arm) expansion. Hormonal therapy and elective nodal RT using simultaneously integrated boost were used at the discretion of the physician, and patients were stratified based on the baseline International Prostate Symptom Score (IPSS) and prostate gland volume. Dosimetric endpoints were collected including Bladder D0.035cc, Bladder V39Gy, Bladder V20Gy, Bladder V40Gy, Urethra Max, and Urethra D0.03cc. The primary endpoint was the incidence of acute grade ≥2 GU toxicity by Common Terminology Criteria for Adverse Events (CTCAE) version 4.03 criteria within the first 90 days after SBRT, which has been reported. The findings of this analysis were that MRI-delivered SBRT is associated with less acute grade ≥2 GU toxicity (7). In total, 156 patients were randomized, and samples from 148 of these participants were available and evaluated at the 2-year follow-up point for this study. Basic patient demographics from those included in this study are reported in Supplementary Table S1. This study followed the Declaration of Helsinki ethical guidelines, written informed consent was obtained from each subject or their guardian on the study, and the study was approved by the UCLA Human Investigation Committee and Institutional Review Board.

Toxicity scoring

In the trial, physician-reported toxicity was evaluated using CTCAE version 4.03 for both acute and late adverse events and included grading for both GU and gastrointestinal toxicities. The scoring assessed the severity of symptoms and their impact on the patient's daily life, providing a standardized method to compare outcomes between the MRI-guided and CT-guided treatment arms. Patients were evaluated at baseline, 1 and 3 months after SBRT, every additional 3 months for the first year after treatment, and then every 6 months through 24 months after SBRT (at the time of a prespecified secondary endpoint analysis). Per the study, as is standard, acute toxicity was defined as grade ≥2 toxicity that occurred within the first 3 months after treatment, and late toxicity as any grade ≥2 that was present after 3 months or later after treatment. There was no distinction between acute toxicity that resolved and that which did not (chronic toxicity), nor was chronic toxicity separated from late toxicity in the trial.

For the purpose of this analysis, a patient was defined as having chronic GU toxicity if they still had the same grade ≥2 GU toxicity that they had acutely 6 months or later after SBRT. Here, we refer to patients with resolving acute GU toxicity as having "acute-only" toxicity, and those experiencing unresolving acute GU toxicity as having "chronic toxicity." We define late GU toxicity as grade ≥2 toxicity that occurred 3 to 6 months or later after treatment, without the occurrence of the same acute grade ≥2 GU toxicity. Therefore, if patients had acute GU toxicity that resolved, but then a unique, second GU toxicity presented at a later timepoint, they were considered to have late GU toxicity in addition to acute GU toxicity, instead of chronic GU toxicity. It was also noted if patients had grade = 2 GU toxicity owing only to α-adrenergic antagonist prescription (or increased dose of a preexisting prescription). Physician scored GU toxicity is reported in Supplementary Table S2.

DNA biomarker panel evaluation and testing

DNA was isolated from cheek swab samples using standard DNA extraction methods (22). Blinded samples had PROSTOX testing performed at MiraDx, per standard operating procedures. MassARRAY genotyping investigating >110 primarily mirSNPs and noncoding variants as previously defined and evaluated (25) was performed on DNA from all samples to further investigate signatures of acute only toxicity and chronic GU toxicity. A small number (<15%) of SNPs in the larger exploratory panel were coding

sequence variants, which were previously identified as potentially important in radiation toxicity.

GU toxicity trend cluster analysis

To attempt to visualize inherent groupings within the dataset based on the trajectories of GU toxicity over time, we employed k-means clustering, using toxicity grade trajectories reported at 3 through 24 months for each patient. Missing GU toxicity grades were imputed with the mean grade at the corresponding timepoint across all patients with at least three timepoints available. Prior to clustering, the toxicity data were normalized. The optimal number of clusters was determined by utilizing the elbow method for the within-cluster sum of squares and identified the most significant change in slope. This analysis suggested a five-cluster solution. Following clustering, the average GU toxicity grade for each cluster at each timepoint was computed. These averages were plotted to visualize and interpret the temporal trajectories of GU toxicity across the identified clusters, and two clusters with patients who did not experience GU toxicity grade ≥2 were combined.

PROSTOX validation

To evaluate PROSTOX's performance in predicting assessed late GU grade ≥2 toxicity, the PROSTOX signature was applied to the relevant mirSNPs in R (RRID: SCR_001905), and values above or equal to 0.5 were classified as high risk (predicted to have late GU toxicity), and those with values below 0.5 were considered low risk (predicted to not have late GU toxicity). Model performance was assessed using sensitivity, specificity, positive predictive value (PPV), negative predictive value, F1 score, and AUC. The treatment arm, α-adrenergic prescription/dose increase as only grade 2 GU toxicity, and pelvic lymph node treatment with simultaneously integrated boost was also evaluated.

Logistic regression was used to assess if other nonbinary clinical variables, including the baseline IPSS, prostate gland volume, use of rectal spacer, as well as whether collected dosimetry factors provided additional information for predicting late GU toxicity beyond that offered by the PROSTOX genetic model. Each measure was analyzed in a separate model, and we used the traditional P value threshold of 0.05 to determine if other variables provided substantial contributions to the prediction of late GU toxicity beyond that of our current genetic PROSTOX biomarker.

Linear regression was used to assess if the PROSTOX probability of late GU toxicity was predictive of GU toxicity grade overall and within treatment arm for patients with no GU toxicity, acute, or late GU toxicity. A Fisher exact test was used to examine whether the PROSTOX signature was predictive of acute or chronic GU toxicity from SBRT.

Prefiltering SNPs to evaluate models of acute-only toxicity and chronic GU toxicity

To develop models to predict grade ≥2 acute-only or chronic GU toxicity, we analyzed outcomes based on the physician scored GU toxicity, which separated people into one of four categories: no toxicity, acute-only toxicity, chronic toxicity, and late toxicity (Supplementary Table S2). To eliminate genetic bias in the development of the model predicting acute-only GU toxicity, we excluded nine individuals who experienced both acute and late GU toxicity.

SNPs were included as potential covariates via sure independent screening (26) to evaluate if they were marginally associated with the outcome of interest (either acute-only or chronic GU toxicity) via the Fisher exact test (27) or Jonckheere–Terpstra test (28) at a P value threshold of <0.2 (exact P values computed via the Monte Carlo method). In the acute-only GU toxicity model, 16 of 111 SNPs were marginally associated to acute toxicity. In the chronic GU toxicity model, 25 of 111 SNPs were marginally associated to chronic toxicity. The proportions of GU toxicity by SNP category for these filtered SNPs are provided in Supplementary Table S3. The choice of the liberal P value threshold is for reducing dimensionality not for associative means. For each SNP genotype, the 0, 1, or 2 count refers to the number of alternative alleles compared with the reference genome.

Developing genetic signatures of acute-only toxicity and chronic GU toxicity

Each prefiltered SNP was coded to obtain up to four possible values: 0, 1, 2, or missing. We fit elastic net (EN; ref. 29), random forest (RF; ref. 30), and boosted tree (BT; RRID: SCR_017301; ref. 31) models to predict toxicity. To minimize sensitivity to imbalances between groups, we used up-sampling to balance the classes (e.g., GU toxicity or not). This process resamples the dataset to include more cases from the minority class while training a predictive model.

Maximizing AUC was used as the training metric for EN and RF models, and minimizing deviance was used for BT models. The optimal λ for EN models were chosen using fivefold cross-validation. For BT models, we used the 0.6 bagging fraction with the Bernoulli distribution. Predicted values above 0.5 were classified as toxicity and equal or below 0.5 as no toxicity. Model performance was assessed using nested leave-one-out cross-validation (LOOCV) sensitivity, specificity, PPV, negative predictive value, F1 score, and AUC. The model with the highest AUC (between all EN, RF, and BT) was chosen for our signature.

Variable importance was calculated as a relative influence compared with the other variables in the model. We calculated the relative influence using a filtering approach, namely, as the relative change in AUC obtained by dropping one variable at a time and comparing to the AUC of the full model. We converted these metrics to a relative scale by dividing the change in AUC by the total change in AUC from all variables.

To understand if there was a contribution of dosimetry planning on the incidence and severity of various GU toxicity outcomes, we employed Kruskal–Wallis and Student t tests to assess differences in six dosimetric measures between individuals who did and did not develop late, acute, or chronic GU toxicity. Subsequently, we examined the correlation between dosimetric measures and the severity of late GU toxicity grades. Finally, to evaluate the relationship between PROSTOX scores and baseline clinical factors, we conducted a correlation analysis using Pearson correlation between the PROSTOX numeric score and pretreatment clinical variables.

Gene Ontology analysis

Gene Ontology (GO) analysis (32) was performed to assess biological pathways that are different between acute-only, chronic, and late GU toxicity signatures. We set the genomic background to the whole genome as this approach was chosen to mitigate the limitations of our SNP panel, which is relatively small (<200) as they were curated based on their hypothesized relevance to cancer outcomes and represent only a fraction of genomic variation. By utilizing the whole genomic background, our analysis was not confined to these preselected variants, thereby allowing us to explore broader biological pathways and possibly uncover additional relevant genes and

processes beyond those initially targeted. To understand biological differences between PROSTOX and preliminary acute-only and chronic GU toxicity signatures, a comparative GO analysis for genes included in the three unique GU toxicity signatures using the universal genomic background at an adjusted *P* value threshold of 0.05 was performed. All analyses were conducted in R (RRID: SCR_001905) using biomaRt and clusterProfiler packages.

Data availability

The raw data generated in this study are available upon reasonable request to the corresponding author. Synthetic data generation and sample code are included in a self-contained Code Ocean compute capsule (https://codeocean.com/capsule/0639380/tree/v1).

Results

Time trend and clustering to visualize temporal patterns of GU toxicity

We performed a clustering analysis to attempt to better visualize the patterns of different forms of RT-associated GU toxicity. The clustering analysis identified five distinct clusters of temporal GU toxicity: no toxicity ($n = 46$), mild toxicity (≤ grade 1, $n = 38$), acute grade ≥2 toxicity ($n = 22$), chronic grade ≥2 toxicity (acute grade ≥2 toxicity not resolving by 6 months, $n = 18$), and late grade ≥2 toxicity (on average, toxicity arising around 1 year, $n = 24$; Supplementary Fig. S1). Given our interest in grade ≥2 GU toxicity specifically, we condensed this into four GU toxicity groups: no significant toxicity, acute toxicity, chronic toxicity, and late toxicity (**Fig. 1**).

Interestingly, we found that the 6-month posttreatment mark emerged as a pivotal timepoint in separating patients who had acute-only GU toxicity from those who had chronic GU toxicity. As expected, late GU toxicity seemed to have a unique temporal pattern that distinguished it from acute-only or chronic GU toxicity. This is the first visualization based on a clustering analysis of these unique forms of GU toxicity. These cluster labels also aligned well with the *a priori* toxicity classifications assigned to patients for this study (none, acute only, late, or chronic) as shown in Supplementary Table S4. Cohen's κ is 0.669, which is considered substantial agreement between the two methods. We therefore used the clinical classifications for PROSTOX validation of late GU toxicity and further evaluation of acute-only toxicity and chronic GU toxicity.

Validation of PROSTOX in MIRAGE and association with grade of GU toxicity

We next evaluated the ability of PROSTOX to predict late grade ≥2 GU toxicity in the MIRAGE trial as a whole as well as across treatment arms (CT or MRI). PROSTOX had strong performance predicting late grade ≥2 GU toxicity with an AUC of 0.762 overall, and similar AUCs across the treatment arms (0.762 in the MRI and 0.761 in the CT-treated groups). Compared with the original training LOOCV metrics, validation in the MIRAGE cohort resulted in similar but slightly higher specificity (0.948 vs. 0.924) and lower overall sensitivity (0.576 vs. 0.714, **Table 1**). Excluding patients who only had a grade ≥2 GU toxicity by virtue of adrenergic antagonist prescription only or increases in bladder dose resulting from pelvic lymph node field inclusion, yielded qualitatively similar results. In addition, PROSTOX performed well in people with or without androgen deprivation therapy (ADT) usage, although it had a slightly better performance in people without ADT usage (PPV = 1.0 vs. 0.727, Supplementary Table S5).

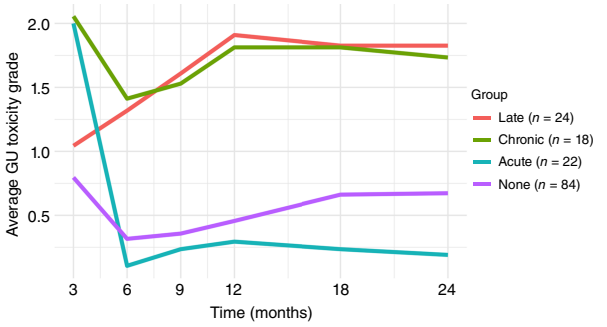

Figure 1.

Average GU toxicity trajectories after SBRT treatment in four grouped clusters. Four groups were made by combining two of the identified clusters. These four categories represent the average GU toxicity grade at each timepoint for people in those groups. There were 24 people in late, 18 in chronic, 22 in acute, and 84 without toxicity.

We also evaluated the association of the numeric output from PROSTOX (as opposed to the binary cutoff value, which indicates toxicity or not) with GU toxicity in each treatment arm. We explored the numeric output predictive power using ROC curves and found that the AUC of PROSTOX was 0.83 in the MRI group and 0.71 in the CT-treated group (**Fig. 2A**). These findings further indicate that PROSTOX can accurately predict grade ≥2 late GU toxicity for both of these SBRT delivery techniques, and if anything, trended to be better for the MRI-treated group.

We next used regression analysis to evaluate if the numeric output of PROSTOX was correlated with the grade of late GU toxicity that patients experienced, which ranged from 0 to 3 in this cohort. We evaluated the distribution of PROSTOX values by treatment arm, including all patients with no toxicity, acute-only toxicity, or late toxicity. We found that a higher PROSTOX score was predictive of higher GU toxicity grade using linear regression. This was true overall (linear regression $P = 1.2 \times 10^{-9}$), in the MRI-treated patients (1.7×10^{-7}), and in the CT-treated patients ($P = 0.001$; **Fig. 2B**; Supplementary Table S6).

Impact of dosimetric and clinical variables on PROSTOX

Next, we evaluated the association between late GU toxicity (both incidence and severity) and clinical and dosimetric measures, which included various bladder and urethra dosages and volumes. We found no significant association between any dosimetric measures and late toxicity risk, but the baseline IPSS was positively correlated with late GU grade, as expected (Supplementary Table S7). In addition, logistic regression models demonstrated the dominance of PROSTOX in predicting late GU toxicity events. After including PROSTOX, no other clinical or dosimetric factors had a significant association with toxicity themselves (**Fig. 3A**) nor improved prediction of late GU toxicity over PROSTOX alone (Supplementary Table S8A; **Fig. 3B**).

In addition, correlation analysis between PROSTOX scores and clinical variables reveal no relationships between them, indicating that PROSTOX is not predictive of clinical factors that may influence GU toxicity. For example, we found a weak, nonsignificant association between PROSTOX scores and baseline IPSS ($r = -0.095$; $P = 0.25$), indicating that PROSTOX predictions are not influenced by baseline urinary symptoms. No other clinical variables demonstrated significant correlations with PROSTOX (Supplementary Table S8B).

Table 1. PROSTOX late GU toxicity signature performance metrics in MIRAGE.

Data	N	Num Tox	Sensitivity	Specificity	PPV	NPV	F1	AUC
MIRAGE overall	148	33	0.576	0.948	0.760	0.886	0.655	0.762
MIRAGE MRI	76	14	0.571	0.952	0.727	0.908	0.640	0.762
MIRAGE CT	72	19	0.579	0.943	0.786	0.862	0.667	0.761
Original signature	93	14	0.714	0.924	0.625	0.948	0.667	0.819

Abbreviation: NPV, negative predictive value; Num Tox, number of people with toxicity.

Genetically modeling acute-only versus chronic GU toxicity

We next tested if we could create signatures that could distinguish patients with significant acute-only versus chronic GU toxicity. We first evaluated the association between acute-only or chronic GU toxicity (incidence) and clinical and dosimetric measures. We found no significant association between any dosimetric or clinical measures and acute-only GU toxicity but found that a higher urethra D0.035cc, higher IPSS, and no spacer use were positively correlated with chronic GU toxicity risk (Supplementary Table S9). We then developed predictive combined models with mirSNPs and clinical and dosimetric variables and compared them with models that used clinical and dosimetric variables only.

The constructed acute-only GU toxicity model had an LOOCV AUC of 0.770 versus an AUC of 0.624 for the clinical- and dosimetric-only model (**Table 2**), indicating that germline genetics are strong predictors of acute GU toxicity. The most important genetics for the acute-only model were three SNPs in *MSH2*, *P2RX7*, and *TGFB1* (Supplementary Table S10), which improved the overall AUC by 0.03 to 0.04 points each and correspond to a relative influence of at least 10%.

The constructed chronic GU toxicity model achieved an LOOCV AUC of 0.763 versus an AUC of 0.654 for the clinical- and dosimetric-only model (**Table 2**). Notably, the most influential predictors of chronic GU toxicity included both clinical factors, such as spacer use and the baseline IPSS, and SNPs in *BMP2* and *IL1A*. Each of these variables had at least 10% relative influence (Supplementary Table S10). This enhancement over clinical- and dosimetric-only models underscores the role of combining genetic factors with clinical parameters to identify predispositions in the case of chronic GU toxicity.

PROSTOX and acute-only or chronic GU toxicity

To assess whether the validated PROSTOX biomarker of late GU toxicity was predictive of other toxicity endpoints, we evaluated its performance in predicting acute-only or chronic GU toxicity. We found that PROSTOX did not predict the occurrence of acute-only or chronic GU toxicity (Supplementary Table S11A). This was further evidenced by area under the ROC curve values, which remained under the nondiscriminatory threshold of 0.5 for chronic and acute-only GU toxicity (Supplementary Table S11B). This finding supports the unique genetic underpinnings of these three forms of RT-associated GU toxicity.

Comparative GO among acute-only, chronic, and late GU toxicity models

We analyzed the GO enrichment pathways associated with our acute-only, chronic, and PROSTOX late GU toxicity signatures, comparing all against a universal genomic background. The GO analysis for acute-only GU toxicity found associations with DNA and RNA regulation, nucleocytoplasmic transport, and cell-cycle regulation. Chronic GU toxicity was enriched in apoptosis and cell-cycle regulation. In contrast, the PROSTOX late toxicity signature primarily involved immune-cell activation and cytokine production,

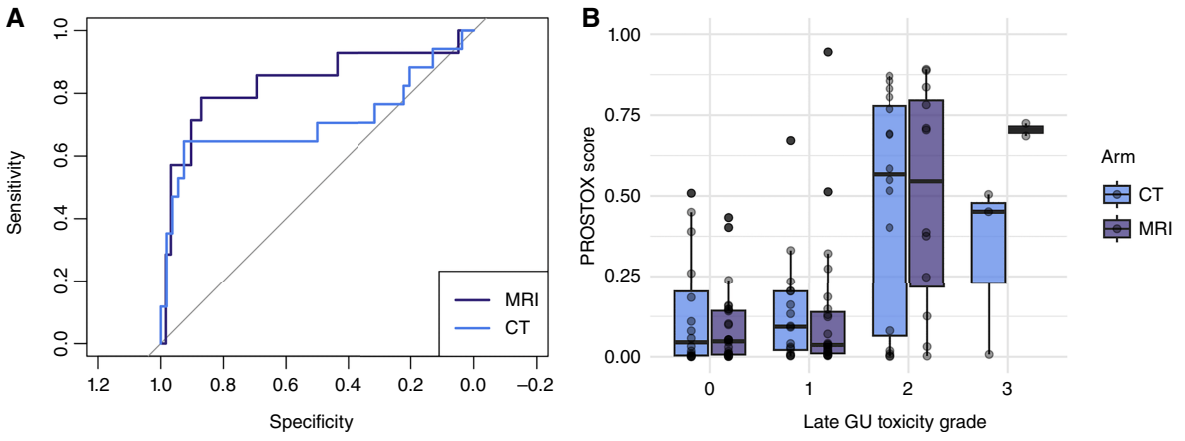

Figure 2.
Validating PROSTOX for predicting late GU toxicity and grade in MIRAGE. **A,** ROC plot displaying PROSTOX accuracy in MRI and CT arms. This displays numeric output from PROSTOX, not its binary classification. The numeric AUCs are 0.83 and 0.71 for MRI and CT, respectively, which differ from the binary AUC presented in **Table 1**. **B,** Boxplot illustrating the relationship between PROSTOX scores and late GU toxicity grades stratified by treatment arm. PROSTOX scores are significantly associated with late GU toxicity grade overall ($P = 1.2 \times 10^{-9}$), within the MRI arm ($P = 1.7 \times 10^{-7}$), and within the CT arm ($P = 0.001$) based on linear regression.

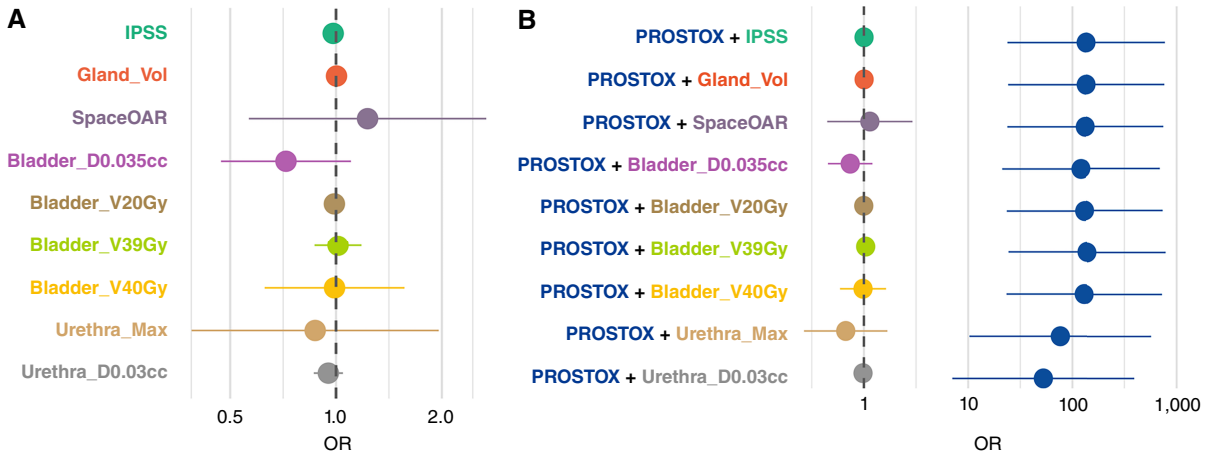

Figure 3.

PROSTOX is predictive of late GU toxicity without benefit from clinical or dosimetric variables. **A,** Forest plot of ORs from logistic regression models evaluating individual clinical and dosimetric factors as predictors of late grade 2+ GU toxicity. Each row represents a model with a single clinical or dosimetric covariate. No factor alone was significantly predictive of late GU toxicity. Error bars represent 95% confidence intervals. **B,** Forest plot of ORs from logistic regression models including PROSTOX and one clinical or dosimetric factor as covariates for predicting late grade 2+ GU toxicity. PROSTOX remains a strong predictor of toxicity across all models, whereas clinical or dosimetric factors do not significantly contribute after adjusting for PROSTOX. Error bars represent 95% confidence intervals. OAR, organs at risk.

highlighting significant immune system dysregulation (Supplementary Table S12; **Fig. 4**).

Discussion

In this study, we validated the PROSTOX biomarker as a predictor of late significant (grade ≥2) GU toxicity in patients treated with SBRT in a clinical trial comparing MRI- versus CT-guided SBRT. Although our analysis was neither of the trial nor meant to compare the technologies themselves, our findings do indicate that PROSTOX predicts late GU toxicity with either treatment modality. We also found that PROSTOX was not improved by the addition of clinical or dosimetric measures, supporting that PROSTOX is not simply reflecting preexisting clinical conditions but instead is likely capturing independent genetic risk factors for late GU toxicity. We also found that the PROSTOX score is correlated with toxicity grade, with a higher score being associated with higher GU toxicity grade. Although it should be acknowledged that the sensitivity of PROSTOX was lower in this study than in the original cohort, this could be explained by the use of a different toxicity scoring systems (CTCAE vs. Radiation Therapy Oncology Group, RTOG) as well as inclusion of pelvic lymph nodes and ADT in this validation study. Finally, we were able to temporally and genetically trisect RT-associated toxicity into acute-only, chronic, and late GU toxicity for the first time. Here, we found that acute-only toxicity and chronic GU toxicity have unique mirSNP-based genetic signatures, and all three forms of toxicity have gene pathway alterations based on GO analyses that differentiate them from each other.

Our GO results identified important differences in the biological processes involved in the unique forms of GU toxicity. In acute-only GU toxicity, the involvement of processes like primary miRNA processing, mitotic DNA damage checkpoint signaling, and regulation of nucleocytoplasmic transport indicates significant changes in cellular state and gene regulation. These processes are likely critical for responding to the acute stress of radiation and correcting DNA damage, ensuring genomic stability and managing cellular response to immediate stress (33–35). The involvement of nuclear transport pathways suggests disruptions in the regulated exchange of molecules between the nucleus and cytoplasm, and dysregulation of these processes may compromise genomic stability and hinder timely DNA repair, contributing to the development of acute GU toxicity. In contrast, chronic GU toxicity was enriched in pathways focused on apoptosis and cell-cycle regulation, which highlight the balance between cell survival and programmed death following SBRT. Both processes point to tissues attempting to modify cell-cycle progression, and dysregulation or genetic factors may lead to unresolving toxicity. Finally, in our PROSTOX late GU toxicity signature, we found enrichment of immune-cell proliferation and cytokine production, highlighting significant immune system dysregulation with possible roles in late fibrosis (36). The enrichment of

Table 2. Acute-only and chronic GU toxicity models' LOOCV performance.

GU toxicity outcome	Variable	Sensitivity	Specificity	PPV	NPV	F1	AUC
Acute only	SNPs + clinical + dosimetric	0.650	0.891	0.500	0.938	0.565	0.770
	Clinical + dosimetric only	0.450	0.798	0.281	0.892	0.346	0.624
Chronic	SNPs + clinical + dosimetric	0.636	0.889	0.500	0.933	0.560	0.763
	Clinical + dosimetric only	0.567	0.741	0.370	0.865	0.447	0.654

Abbreviation: NPV, negative predictive value.

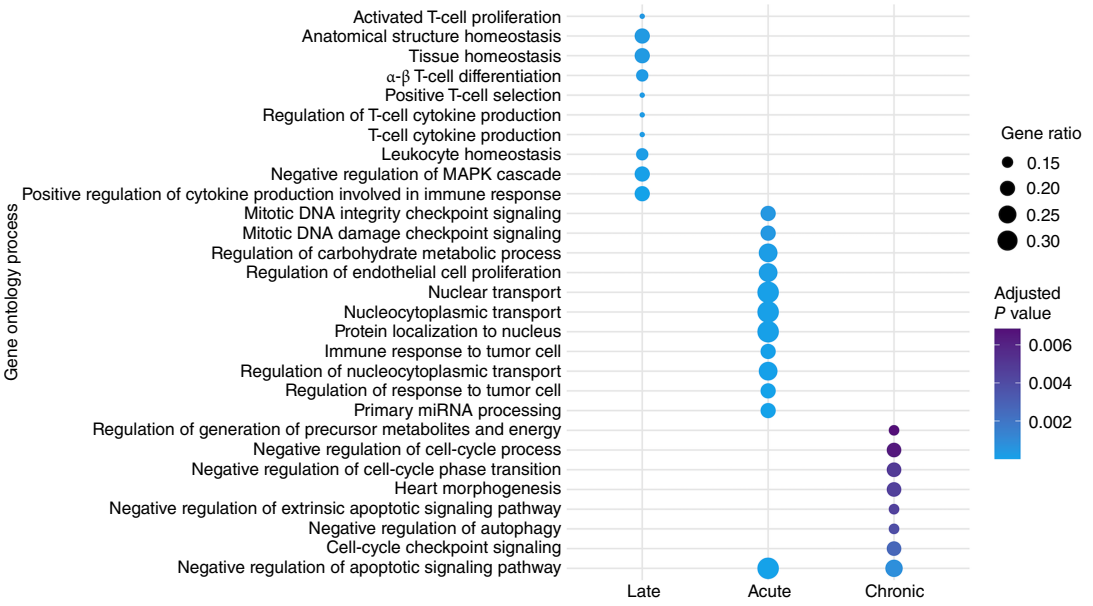

Figure 4.

Top GO enriched pathways involved in PROSTOX (late), acute-only, and chronic GU toxicity. PROSTOX is enriched in immune response regulation and cytokine production, whereas acute only is enriched in protein transport and DNA checkpoints, and chronic GU toxicity is enriched in cell-cycle and apoptotic processes.

these processes suggests that late GU toxicity may be driven by the activation and differentiation of T cells, contributing to inflammation and tissue damage after radiation, as previously hypothesized (37–39). The negative regulation of the MAPK cascade, a key signaling pathway involved in cellular responses to stress and inflammation (33), suggests that MAPK signaling, known to be critical in RT damage (40), may also contribute to the persistence of toxic effects in tissues exposed to radiation. Although the use of a universal genomic background in our GO analysis was intended to mitigate bias introduced by the curated SNP panel, we acknowledge that the initial selection of SNPs focused on cancer-related processes may still influence the enrichment of certain biological pathways. This preselection bias may overrepresent pathways already implicated in oncologic outcomes, and, thus, our GO analysis findings should be interpreted as exploratory and will require further validation in broader, unbiased genomic datasets.

Other limitations of our study include a small sample size, preventing conclusions on the true impact of technology on these unique forms of toxicity. In addition, in this study, we newly defined and modeled chronic RT-induced toxicity, which is not currently separately reported in clinical trials or in the MIRAGE trial, making a direct comparison of our results with reported results of reduced toxicity with MRI treatment not possible. We acknowledge that thoughtful evaluation will need to be done to best characterize chronic toxicity moving forward. Although we are not proposing that current clinical toxicity scoring be revamped to separately identify chronic toxicity, we believe that our findings raise some important considerations about future toxicity analyses. First, because chronic GU toxicity seems to be a separate genetic entity from late GU toxicity, it should be recognized when attempting to genetically identify those at increased risk of long-lasting RT toxicity. In addition, based on the importance of clinical and dosimetric measures in addition to genetics predicting chronic GU toxicity in this analysis, it may be that chronic toxicity is the form of radiation toxicity most reduced by advanced technological delivery.

We acknowledge that numerous germline genetic studies have been performed to identify biomarkers of RT-associated toxicity in prostate cancer, including genome-wide association studies (GWAS; ref. 16). A GWAS investigating long-lasting low-grade urinary toxicity for patients with prostate cancer (17) that included more than 3,000 patients identified seven SNPs associated with long-lasting RT toxicity. Our study incorporated three of these variants, but our panel also captured many additional mirSNPs not found in current GWAS arrays, allowing expansion of the scope of genetic factors linked to RT toxicity. Interestingly, the most important GWAS SNP, which is also part of the PROSTOX biomarker panel, disrupts a noncoding RNA (41), further highlighting the importance and relevance of evolving genetic arrays to capture functional noncoding elements (42–44). Other important differences between our study and GWASs, beyond our separation of late GU toxicity as a unique entity from chronic toxicity, include the following: our studies exclusively included modern SBRT techniques versus varied treatment approaches, based on our prior evidence for unique genetic signatures of toxicity based on fractionation (22); and we have applied advanced statistical methods, like EN and BT methods, to address the complexities of SNP interdependencies, leading to superior model performance with an AUC of 0.76, far exceeding the modest discrimination achieved by previous GWAS models, with a top C statistic of 0.621 (18).

Our findings indicate that to continue to decrease significant RT-induced long-lasting GU toxicity in patients with prostate cancer, both genetic identification of those at risk in conjunction with continued technological development is necessary. PROSTOX is validated here to identify patients at genetic risk of late significant GU toxicity, affording them the opportunity to choose alternative, safer treatment approaches, of which there are several options, including CFRT (as described in our prior study; ref. 22). However,

PROSTOX does not identify patients at risk of chronic GU toxicity, which may be potentially decreased through the application of advanced technology. As both late toxicity and chronic GU toxicity significantly affect a patient's quality of life (45) and pose a significant financial burden to the medical system as a whole (46, 47), the importance of reducing both of these forms of toxicity is most pressing.

Our findings represent critical steps toward meaningfully identifying patients as genetically radiosensitive, which will enable appropriate treatment selection for the best outcomes. Ongoing work continues to validate mirSNP-based panels as toxicity and outcome predictors in other cancer types treated with radiation, as well as across other cancer therapies. Finally, with further validation of our GO findings, this study provides a foundation for future work to explore potential targeted and precision-based therapeutic interventions to prevent late RT-associated GU toxicity, with the ultimate goal to improve patient outcomes and quality of life.

Authors' Disclosures

A.U. Kishan reports grants from the University of California Cancer Research Coordinating Committee, Department of Defense, and NIH during the conduct of the study, as well as grants and personal fees from Varian Medical Systems, Inc., Janssen, and Lantheus, personal fees from Boston Scientific and Novartis, and grants from POINT Biopharma and ArteraAI outside the submitted work. M. Cao reports personal fees from Varian outside the submitted work. J.B. Weidhaas reports grants from the NIH and nonfinancial support from MiraDx during the conduct of the study; other support from MiraDx outside the submitted work; a patent for miRNA mutations at Yale University licensed to MiraDx; and being a co-founder of MiraDx, which has developed the PROSTOX test. No disclosures were reported by the other authors.

Authors' Contributions

A.U. Kishan: Conceptualization, formal analysis, methodology, writing–original draft, writing–review and editing. **K. McGreevy:** Formal analysis, methodology, writing–original draft, writing–review and editing. **L. Valle:** Data curation, writing–review and editing. **M. Steinberg:** Conceptualization, data curation, writing–review and editing. **B. Neilsen:** Data curation, writing–review and editing. **M. Casado:** Data curation, writing–review and editing. **M. Cao:** Data curation, writing–review and editing. **D. Telesca:** Methodology, writing–review and editing. **J.B. Weidhaas:** Conceptualization, data curation, formal analysis, funding acquisition, validation, methodology, writing–original draft, writing–review and editing.

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Note

Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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