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Exploratory study of a *KLK2* polymorphism as a prognostic marker in prostate cancer

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Abstract

Objectives—An association of a single nucleotide polymorphism (SNP) of the *KLK2* gene (rs198977; c.748C>T; R250W) with risk for developing prostate cancer has been observed. We evaluated the role of R250W SNP for prognosis in prostate cancer.

Methods—The c.748C>T SNP was genotyped from blood DNA of 182 patients after completing initial cancer treatments. For evaluating prognosis of genotype groups, associations were performed with Gleason score (GS) and biochemical recurrence free survival (bRFS) in patients demonstrating PSA-recurrence after initial cancer therapy.

Results—Overall distribution of the CC, CT and TT genotypes for the SNP was 48%, 44% and 8%, respectively. The distribution of high (8–10), moderate (5–7) and low (2–4) GS among the genotype groups was 17%, 74% and 9% for CC group compared to 25%, 74% and 1% for the CT/TT ($P = 0.04$). Median bRFS time for CT/TT group was 36.5 months compared to 44.5 months for the CC group ($P = 0.16$), while genotype groups combined with morphology revealed significantly different bRFS ($P = 0.004$).

Conclusions—This exploratory analysis in prostate cancer patients revealed the W allele of the *KLK2* R250W SNP to be less likely associated with low GS morphology. Further studies will be needed to confirm this observation in larger cohorts.

Keywords

Prostatic neoplasm; kallikrein; polymorphism; prognosis

1. Introduction

Prostate cancer is the most common malignancy in US males and the second most common cause of cancer related mortality accounting for 27,360 deaths in 2009 [1]. In the past

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decade, incremental gains have resulted in increasing cancer specific survival and decreasing cancer related morbidity and mortality. Cancer management strategies that may have resulted in these gains include earlier diagnosis of organ-confined cancer, the advent of multi-modality combination treatments for non-localized tumor stage [2] and new chemotherapy regimens for advanced stage disease [3,4]. At present it remains debatable whether the widespread use of prostate specific antigen (PSA) as a screening test for cancer diagnosis unequivocally contributes to reduction in cancer specific mortality [5–8]. PSA is a serine protease, which is also extensively used for post treatment monitoring of disease recurrence and for evaluating response to cancer treatments [9]. It is the most well known member of the kallikrein family which is the largest contiguous group of proteases in the human genome clustered in a 300-kb region on chromosome 19q13.4. The kallikrein family consists of 15 genes all of which have 5 coding exons and share significant homology at the DNA and amino acid level. Human kallikrein-3 (*KLK3*), better known as PSA, and human *KLK2* are closely related sharing the highest homology amongst all kallikreins with 78% to 80% identity at the amino acid and DNA levels respectively [10]. While both kallikreins are found predominantly in the prostate gland, *KLK2* concentration in serum is only 1-3% of PSA [11]. Unlike PSA, which lacks sensitivity for identifying tumor grade and stage in newly detected cancers, serum *KLK2* levels correlate with tumor stage, degree of differentiation and total tumor volume thereby enhancing its prognostic value [12–16]. The ability to distinguish poorly differentiated (Gleason 4/5) tumors and to predict pathologically organ confined cancer makes serum *KLK2* a potentially important prognostic marker. In addition to the predictive value of serum *KLK2* levels, a single nucleotide polymorphisms (SNP) in the *KLK2* gene, c.748C>T, has been found to be associated with prostate cancer risk. Taken together, the combination of the specific *KLK2* polymorphisms and circulating *KLK2* appear to enhance prediction of prostate cancer risk [17]. The T allele of the c. 748C>T polymorphism encodes a variant protein with tryptophan (W) instead of arginine (R) at position 250 in the primary translation product (position 226 in the mature protein). This 250 W form lacks the trypsin-like proteolytic activity found for the wild type protein [16] and has been associated with an increased risk of prostate cancer (OR: 1.51 for CT and 2.13 for TT) [17], although the impact of the presence the variant protein may have on long-term prognosis in patients diagnosed with prostate cancer is not known.

We hypothesized that the T allele of the R250W polymorphism will adversely impact prognosis in prostate cancer patients. To evaluate this, in a pilot study we determined the genotype for the polymorphism in a series of 182 prostate cancer patients visiting a tertiary level academic hospital and determined the association of genotype groups with and without the T allele with Gleason score (GS) at diagnosis, a well accepted prognostic marker in prostate cancer. We then evaluated the biochemical relapse free survival (bRFS), a surrogate marker for long-term prognostic outcome, among the genotype groups with-in this cohort in patients who experienced PSA relapse after initial cancer treatments.

2. Materials and methods

2.1. Study subjects

A collection of blood specimens for prospective biomarker research from prostate cancer patients visiting ambulatory clinics within a single university was performed after obtaining written informed consent on an institutional review board approved study initiated in 2006. Research subjects included newly diagnosed prostate cancer patients and previously diagnosed and treated prostate cancer patients in localized or advanced cancer stages returning for clinical follow up who gave voluntary approval to access previous and/or future clinico-pathological records necessary for conducting research. Clinical follow-up included the current standard of care used to diagnose, treat and follow up prostate cancer

patients such as clinical examination, periodic and serial PSA measurements during visits and imaging scans whenever necessary, guided by physician recommendations. Clinico-pathological data collected from medical records and follow up on study subjects included patient demographics; histopathology and morphology (Gleason Score-GS); presenting PSA, types of primary prostate therapy delivered and time to PSA recurrence after initial cancer treatment as evidenced by a serial rise in PSA on two separate occasions more than four weeks apart. Where available, retrospective data was also collected using the updated Phoenix definition for biochemical failure in patients receiving radiation alone.

2.2. Genotype analysis

DNA was purified from peripheral blood using the QIAamp Blood DNA extraction kit (Qiagen Inc., Valencia, CA). Genotype analysis of the c.748C>T polymorphism (rs198977) was done using PCR followed by differential melting of an oligo probe labeled with fluorescein. The nucleobase quenching phenomenon was used to detect probe hybridization [18,19] The PCR and acquisition of fluorescence data was done in the Roche LightCycler (Basel, Switzerland). The sequences of the primers were 2KLK-1: 5'-CCTGCCTGAAAAGCCTGCTGTGTA and 2KLK-2: GTGATGCCAGAACGTGAGGTGGAC. The probe sequence was 5'-CACTTCCAGTAATGCACCAA, labeled on the 5' terminus with 6-FAM and blocked from extension by the polymerase with a C3-linker on the 3' terminus. The PCR was performed in LightCycler capillary tubes in a volume of 20 μl with Roche Fast-Start DNA Master Hybridization Probes, $MgCl_2$ to achieve a final concentration of 2.2 mM, 1 μM 2KLK-1, 0.25 μM KLK-2-2, and 0.2 μM. probe. Primers and probe were purchased from Integrated DNA Technologies (Coralville, IA). The reaction was heated to 95°C for 10 minutes to activate the hot start Taq DNA polymerase, then cycled 60 times between 95°C for 2 seconds, 66°C for 2 seconds and 72°C for 6 seconds, with ramping rates at the maximum of 20°C/second except for the 66°C to 72°C transition which was done at 1°C/second. After PCR the melting curve thermal profile was 95°C for 10 seconds, 40°C for one minute, then 75°C with a 0.1°C/second temperature transition rate while continuously collecting fluorescence data. The probe was matched to the c.748T allele (250W) giving a melting temperature (Tm) of 56.6°C for 250 W homozygotes. The c.748C allele (encoding 250R) gives a lower Tm of 48.3°C due to the single base mismatch between the PCR product and the probe. Heterozygotes give signals at both Tms indicating the presence of both alleles.

2.3. Data analysis

The Kaplan-Meier method was utilized to estimate survival curve of bRFS in each group. The log-rank test was used to compare survival curves between different groups. Fisher's exact was used to compare genotype and group frequencies. All p-values were calculated based on two-sided tests with significance level set at 0.05. The statistical analyses were implemented with SAS 9.1.3 (SAS Institute Inc., Cary, NC).To evaluate prognostic value of the *KLK2* c.748C>T polymorphism two clinically accepted prostate cancer specific prognostic variables were chosen for association with genotypes. The frequency of the wild type (C allele) and the variant allele (T) among high (8–10), moderate (5–7) or low (2–4) degree of differentiation (graded by Gleason score) was evaluated using Fisher's exact test. The second prognostic variable, biochemical relapse free survival (bRFS) period for the study population was obtained during active follow up of patients after enrollment and/or retrospectively from patient records. The bRFS period was defined as time from date of initial prostate cancer treatment to the date of a serially rising PSA on two separate occasions four weeks apart, after a post-treatment nadir, with the date of the second serially rising measurement considered as biochemical failure. In this pilot study, genotypes with any variant allele (single or double T allele) were analyzed as one group as were moderate and low grade GS patients (Gleason Score: 2–7) for survival analyses. Log-rank test was

used for comparing the bRFS time in the high and moderate/low GS groups and between CC and CT/TT genotype groups. bRFS was also compared after combining genotype groups (CC vs. CT/TT) with Gleason score groups (high vs. moderate to low) using log-rank test for determining whether the addition of genotype groups to morphology based traditional poor and moderate to well differentiated adenocarcinoma could predict prognosis with-in high and moderate/low GS subsets of patients. Finally, univariate and multivariate Cox regression analysis was performed using covariates of GS, genotype group and initial prostate cancer treatments given (radical prostatectomy, radiation alone or radiation combined with hormonal therapy) to evaluate effect on bRFS.

3. Results

A series of one hundred and eighty three prostate cancer patients visiting Urology and Oncology clinics in a single institution volunteered participation and were genotyped for the c.748C>T polymorphism. One patient withdrew consent after genotyping and was removed from final data analyses. Table 1 provides the study population characteristics. Gleason score information obtained from medical records was available on 169/182 research subjects, either from the post prostatectomy specimen or from the initial diagnostic biopsy in patients not undergoing prostatectomy (distribution of Gleason score in the 169 patients is provided in Table 1). The median follow-up period obtained from medical records for the study population from time of initial cancer treatment to date of analyses was 55.8 months (Table 1) during which 87 patients were found to have experienced a biochemical failure (as defined above) after reaching a post treatment PSA nadir. For the study population experiencing a biochemical recurrence, median PSA at the time of relapse was 2.30 ng/ml (IQR 0.45–7.82 ng/ml) over a mean period of 49.3 months (IQR 14.2–66.0 months).

3.1. Relationship between genotype, Gleason Score and bRFS survival

The *KLK2* c.748C>T genotype distribution in the 182 patient study population was 48% CC, 44% CT and 8% TT. The genotype distribution was in Hardy-Weinberg equilibrium (P = 0.9, chi-square test) and was also very similar (P = 0.7, chi-square test) to the prostate cancer cohort described by Nam et al. [17].

The distribution of high (8–10), moderate (5–7) and low GS (2–4) among the CC and CT/TT genotype groups was 14 (17%), 61 (74%) and 7 (9%) compared to 22 (25%), 64 (74%) and 1 (1%) respectively (Fisher's exact test P = 0.04). The median bRFS time for the high GS (8-10) group was 25.4 months compared to 44.3 months for moderate/low GS (</=7) (Table 2) (log-rank test P = 0.001). The median bRFS time for patients with CT/TT genotype was 36.5 months compared to 44.5 months for the CC group (Table 2). Figure 1 shows the Kaplan-Meier survival curves based on genotype groups. The effect on bRFS with the combination of genotype groups (with and without a T allele) and high (8–10) or moderate to low (2 to 7) GS is presented in Table 3 and Fig. 2 with a significant difference among groups (overall log-rank test among these four groups; P = 0.004).

We also evaluated the effect of Gleason score, genotype distribution and initial cancer treatments received by the study population on bRFS. Of the 112 patients who underwent initial radical prostatectomy (82 patients, and 30 with radical prostatectomy followed by adjuvant post-op radiation GS was available on 101 patients. Of the 62 patients undergoing either radiation alone or combined with hormonal treatments or hormonal treatments alone (15 patients, 26 patients, and 21 patients respectively) GS was available on 55 patients. In evaluating effect of initial cancer treatments on bRFS, univariate and multivariate regression analysis was performed with each unique covariate (initial treatment, Gleason score, and genotype group) in a Cox regression model. The hazard ratio (HR), 95% confidence interval of HR for the multivariate analysis is reported in Table 4. GS alone was observed to be

associated with a higher risk for recurrence (Hazard ratio 2.072, $P = 0.006$) with no impact on survival observed based on type of cancer treatments delivered.

4. Discussion

Numerous nomograms and prognostication models for prostate cancer outcomes currently in use are based on somatic tumor characteristics including Gleason score, tumor stage and PSA levels for predicting pathological stage of disease [20] and/or long-term clinical outcomes including progression-free survival and cancer specific mortality [21,22]. Since these models incorporate morphological and clinical variables there is in-built subjectivity which affects long-term predictions. For example, there is a greater than 24% chance of tumor under staging and under grading of newly diagnosed prostate tumors even in academic centers of excellence [23] which injects inaccuracy and bias while estimating outcomes. Additionally, PSA measurements are assay and lab dependent leading to inter-laboratory variability which also impacts prognostication. Finally, prognostication and prediction nomograms in prostate cancer are treatment specific (radical prostatectomy versus radiation) and may not be universally applicable to patient populations undergoing multi-modality initial cancer therapeutic interventions. Despite these shortcomings the current models have seen extensive use in clinical practice both for deciding cancer treatments and counseling patients.

To overcome these subjective shortcomings, exploration of host genetic markers among cancer patients appears an attractive but under explored option. The incorporation of inherited germline marker based prognosis has attracted recent attention in several other tumor types on the premise that these markers will reflect the natural history of disease progression regardless of cancer treatments as they are likely to be implicated in the continuum from cancer causation, recurrence to mortality. Variation among several inherited genetic markers have been reported for the prediction and/or prognostication of prostate cancer clinical outcomes including polymorphisms with-in the CYP3A4 [24], AR [25,26] and SRD5A2 [27].

A pre-requisite for exploring inherited genetic markers for cancer outcomes is the presence of an *a priori* functional or biological significance for the chosen candidate marker for exploration. In this context *hKLK2* may have a pertinent role to play in prostate cancer prognosis as it has been demonstrated in prostate tissue to convert the inactive form of PSA, pro-PSA into active PSA [28] and the serum level of h*KLK2* has been shown to be associated with other aspects of prostate cancer biology [12–16,29]. For these reasons we considered h*KLK2* as a candidate for exploring the relationship of a measured functional genomic variant with the course of disease. The present study found that the presence of the W allele of the R250W polymorphism is less likely to be associated with well differentiated tumors (low Gleason score), but is more likely to have a shorter bRFS interval. We recognize that biochemical failure in prostate cancer follow-up after primary treatment is at best an intermediate endpoint for long-term survival since the natural history of a PSA relapse can be diverse based upon heterogeneous tumor biology [22, 30]. Thus while it may not necessarily be adequate for predicting death from prostate cancer, it is reported as a surrogate endpoint in early explorative trials and in phase II prostate cancer intervention clinical trials.

Not surprisingly in the analyses, men with less aggressive cancer morphology (low or moderate GS) fared better than those with high-GS. The presence of the W allele (genotypes CT and TT), was observed to have an association with aggressive morphology compared to patients with homozygous R (genotype CC) (Fig. 1), and while survival was inferior for patients carrying the W allele it did not reach statistical significance ($P = 0.16$; Fig. 1).

Interestingly, when genotype group based survival was combined with morphology, sub-groups within the morphology based prognostic model of high GS group patients with a CT or TT genotype had a shortened bRFS (25.4 months for a T allele and 37.5 months for patients with a C allele), which did not reach statistical significance ($P = 0.16$). This observation however is based on a small number of patients with high Gleason score ($N = 36$) with the W allele ($n = 22$) and without ($N = 14$).

5. Conclusions

This exploratory observation for the *KLK2*, R250W polymorphism identifies a potential biologically relevant host genome candidate that could enhance existing prognostic morphology based models in prostate cancer. The limitations of an uncontrolled retrospective small series however require prospective validation in larger cancer cohorts with longer term survival data that includes disease specific progression events including overall and cancer specific survival while taking into account inter-ethnic variability, assay reproducibility, replication and consistency across studies prior to its clinical application as a genomic classifier of prognosis.

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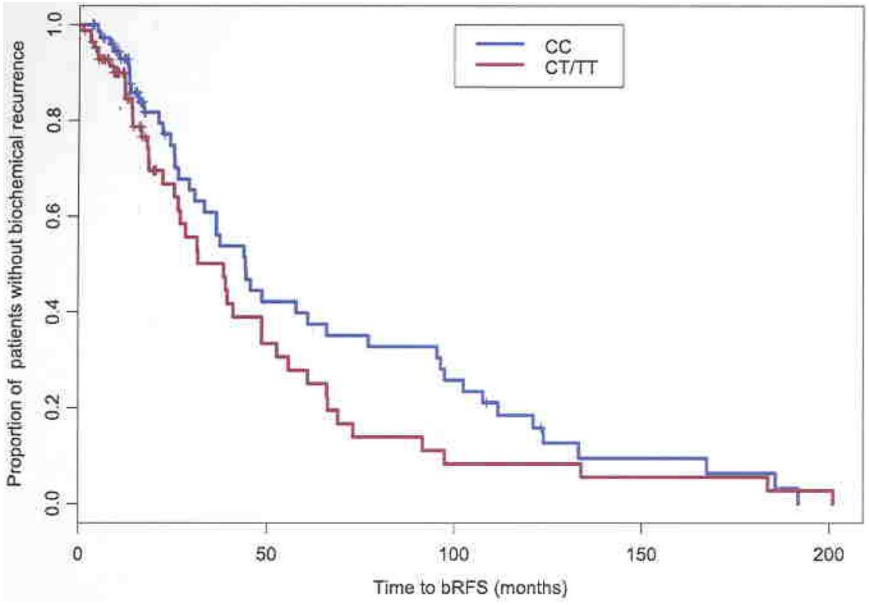

Fig. 1.

Biochemical Relapse Free Survival (bRFS) Analysis for Genotype Groups (log-rank test p-value = 0.16, censored times marked by "+").

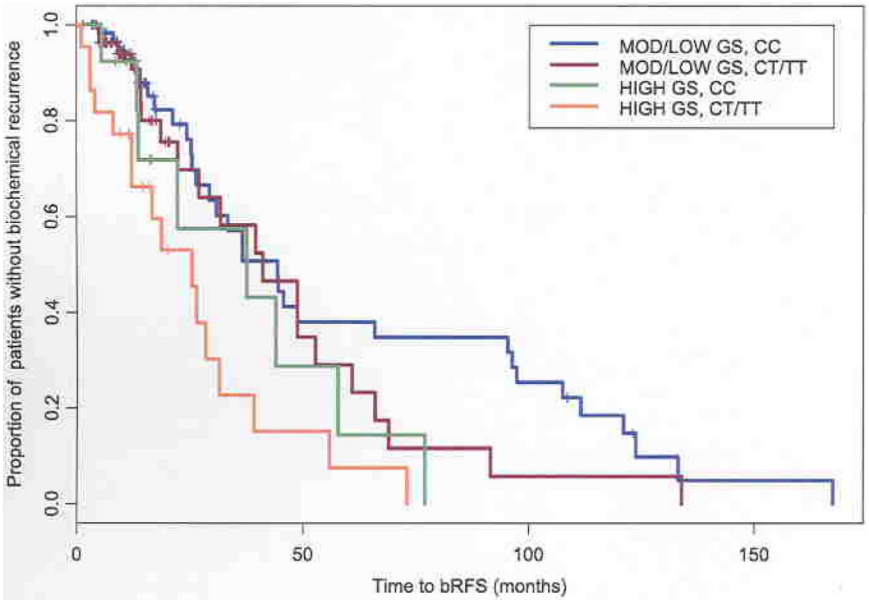

Fig. 2.

Survival analysis for Gleason Score combined with genotype groups (log-rank test *p*-value = 0.004, censored times marked by "+").

Table 1

Study population characteristics

Race	
Caucasian	178 (97.8%)
African American	3 (1.6%)
East Indian	1 (0.5%)
Age (years)	Median 69.0 years, IRQ 42.0–90.0 years
Initial Prostate Cancer treatment	
Prostatectomy	82 (45.1%)
Prostatectomy + Radiation	30 (16.5%)
Radiation	15 (8.2%)
Radiation + ADT	26 (14.3%)
ADT	21 (11.5%)
Observation only	8 (4.4%)
Gleason score	169/182
High	36 (21.3%)
Moderate	125 (74.0%)
Low	8 (4.7%)
Follow-up time (months)	Median 55.8, IRQ 11.5–81.6
Biochemical failure	87/182 (47.8%)

Table 2

Survival analysis for Gleason Score and genotype groups

		N	Median time to biochemical relapse (months)	Log-rank test p-value
Gleason Score	Low/Moderate (2–7)	133	44.3	0.001
	High (8–10)	36	25.4	
Genotype groups	CC	87	44.5	0.16
	CT/TT	95	36.5	

Table 3

Survival analysis for Gleason Score combined with genotype groups

Group		N	Median time to biochemical relapse (months)	Log-rank test p-value
Gleason score	**Genotype**			
Low/Moderate (2–7)	CC	68	44.3	0.30
	CT/TT	65	41.1	
High (8–10)	CC	14	37.5	0.16
	CT/TT	22	25.4	

The overall p-value (from log-rank test) of comparison among 4 groups is 0.004.

Table 4

Multiple Cox regression analysis with initial treatment, GS and genotype group as covariates

Covariates	Hazard ratio	95% confidence interval	p-value
S+SX vs. other treatments	0.861	0.541–1.371	0.53
High vs. Med/low GS	2.072	1.236–3.473	0.006
CC vs. CT genotype	0.686	0.427–1.102	0.12

S: Surgery-Radical Prostatectomy; X: Radiation.