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Original research article

Prognostic correlation of cell cycle progression score and Ki-67 as a predictor of aggressiveness, biochemical failure, and mortality in men with high-risk prostate cancer treated with external beam radiation therapy☆

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ABSTRACT

Objectives: Ki-67 is a proliferation marker in prostate cancer. A prognostic RNA signature was developed to characterize prostate cancer aggressiveness.

The aim was to evaluate prognostic correlation of CCP and Ki-67 with biochemical failure (BF), and survival in high-risk prostate cancer patients (pts) treated with radiation therapy (RT).

Methods: CCP score and Ki-67 were derived retrospectively from pre-treatment paraffin-embedded prostate cancer tissue of 33 men diagnosed from 2002 to 2006.

Abbreviations: pts, patients; IHC, immunohistochemical; HE, hematoxylin and eosin; CCP, cell cycle progression; ADT, androgen deprivation therapy; FFbF, freedom from biochemical failure; OS, overall survival; GU, genitourinary; GI, gastrointestinal; CTCv3.0, common terminology criteria for adverse events; CTV, clinical target volume; DFS, disease-free survival; DMFS, distant metastasis free survival; CSS, cause-specific survival; PCa, prostate cancer; PSA, prostate specific antigen; Gy, gray; RT, radiation therapy.

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CCP score was calculated as an average expression of 31 CCP genes. Ki-67 was determined by IHC. Single pathologist evaluated all tissues. Factors associated to failure and survival were analyzed.

Results: Median CCP score was 0.9 (-0-1 – 2.6). CCP 0: 1 pt; CCP 1: 19 pts; CCP 2: 13 pts. Median Ki-67 was 8.9. Ki-67 cutpoint was 15.08%.

BF and DSM were observed in 21% and 9%. Ki-67 ≥ 15% predicted BF ($p = 0.043$). With a median follow-up of 8.4 years, 10-year BF, OS, DM and DSM for CCP 1 vs. CCP 2 was 76–71% ($p = 0.83$), 83–73% ($p = 0.86$), 89–85% ($p = 0.84$), and 94–78% ($p = 0.66$).

On univariate, high Ki-67 was correlated with BF ($p = 0.013$), OS ($p = 0.023$), DM ($p = 0.007$), and DSM ($p = 0.01$). On Cox MVA, high Ki-67 had a BF trend ($p = 0.063$). High CCP score was not correlated with DSM.

Conclusions: High Ki-67 significantly predicted outcome and provided prognostic information. CCP score may improve accuracy stratification. We did not provide prognostic correlation of CCP and DSM. It should be validated in a larger cohort of pts.

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1. Background

The standard-of-care treatment in high-risk prostate cancer (PCa) patients (pts) is radiation (RT) and androgen deprivation therapy (ADT). Approximately 30–50% of these pts failed biochemically after radical RT at 10 years of follow-up.[1] High-risk group PCa pts have heterogeneous clinical outcomes. Even though the currently used clinical factors (clinical T stage, serum prostate-specific antigen (PSA) level, and Gleason score) and prognostic models[2] provide a useful prognostic outcome information, they are still insufficient to identify who will fail and die. Identifying them at diagnosis might benefit optimal therapy. Nowadays, there is an increasing evidence that some molecular biomarkers and cell cycle expression genes may potentially provide a more accurate prognostic information related to clinical outcomes and to characterized prostate cancer aggressiveness.[3,4]

One promising immunohistochemical (IHC) biomarker is Ki-67, a cell proliferating marker. Ki-67 antigen is a nuclear protein complex, detectable by MIB-1 antibodies. Ki-67 antigen is present in all active phases of the cell cycle (except in the G0 phase), making it a marker of cellular proliferative activity. Previous studies have identified Ki-67 as a biomarker in PCa.[5–7]

Recently, a RNA signature from tumour tissue has been developed to provide additional prognostic information on PCa. A 31-gene signature basically determines the expression level of cell cycle progression (CCP) and measures the fraction of tumour cells that actively divide. The CCP score has been shown to be an independent prognostic information in the setting of RT, observation, and prostatectomy.[8–10]

CCP score and Ki-67 give individually potential prognostic information of cell cycle on PCa pts, but their ability to predict together the outcome on PCa pts after RT is untested. We examined the prognostic correlation of the CCP score and expression of Ki-67 with PCa aggressiveness, biochemical failure (BF) and disease-specific mortality (DSM) in high-risk PCa pts treated with RT.

2. Methods and materials

2.1. Patient characteristics

Patients with clinically localized PCa, and local biopsy-proven high-risk adenocarcinoma, PSA ≥ 20 ng/ml or Gleason ≥ 4 + 4 or cT3N0 or two intermediate factors,[11] and treated with definitive RT in combination with ADT were included in this pooled-analysis study. Mean age was 68 years at the time of diagnosis. More than 50% of pts had cT3, mean PSA was 19 ng/ml, and 48.3% of pts had Gleason score 4 + 3. Median CCP score was 0.8 (Table 1).

Patients were excluded if they had not available formalin-fixed and paraffin-embedded blocks containing their original diagnostic biopsy. Patients with pre-treatment PSA values greater than 100 ng/ml were excluded as likely to have metastatic disease.

CCP score and Ki-67 were derived retrospectively from pre-treatment paraffin-embedded prostate cancer tissue of 33 men diagnosed from 2002 to 2006.

All participating patients gave their written informed consent.

2.2. Treatment characteristics

The prescription of RT dose was 46 Gy (2.0 Gy/d 5 times a week for 23 fractions) to the prostate and regional lymphatics, followed by 30 Gy (2.0 Gy/d 5 times a week for 15 fractions) for a total of 76 Gy to the seminal vesicle and prostate. ADT begun 2 months before RT and was continued during and after RT for a total of 24 months. Total ADT was accomplished with bicalutamide 50 mg/d plus a luteinizing hormone-releasing hormone agonist.

2.3. Evaluation of Ki-67 protein expression by immunohistochemistry

Immunohistochemistry analysis was performed in all cases on formalin-fixed, paraffin-embedded prostate core needle

Table 1 – Pretreatment characteristics of patients.

Characteristic	*n*	Summary measure
CCP score, median (IQR)	33	0.8 (0.5, 1.2)
CCP risk of aggressiveness (%)	33	
Consistent (level 0)	5	15
More aggressive (level 1)	23	70
Considerably more aggressive (level 2)	5	15
Ki-67, median (IQR)	33	6.8 (4.8, 10.6)
Age at diagnosis, median (IQR)	33	70 (67, 72)
Baseline PSA[a] (ng/ml), median (IQR)	33	15 (8.9, 24)
Gleason score (%)		
<7	6	18.2
7	17	5.5
>7	10	30.3
Percent positive cores (%)		
<50	12	36.3
≥50	21	63.7
Percent total cores		
<6	26	79
≥6	7	21
Perineural invasion		
Yes	8	24
No	25	76
ADT (%)		
Yes	28	84.9
No	5	15.1
Radiation dose (Gy), median (IQR)	33	76 (74, 78)

CCP = cell cycle progression; IQR = interquartile range; PSA = prostate-specific antigen; ADT = androgen deprivation therapy; Gy = Grey.
[a] Maximun baseline PSA level was 76 ng/mL.

biopsy tissues. A 4-μm section was stained using the EnVision kit (DAKO, Glostrup, Denmark) and the autostaining system (Dako, Autostainerlink 48, Denmark) with the monoclonal mouse anti-human Ki-67 prediluted, clone MIB1 (DAKO, Glostrup, Denmark). All sections were visualized with the diaminobenzidine reaction and counterstained with haematoxylin. Prostate carcinoma was considered positive if any nuclear staining, regardless of intensity, was observed for Ki-67. One investigator reviewed the slides under a light microscope and with an image analysis system (Cell B imaging software, Olympus, Germany). For manual analysis, the Ki-67 expression was defined as the percentage of tumour cells that displayed nuclear MIB-1 staining. Whenever possible, 2000 o more tumour cells were counted. For cell B imaging analysis, the percentages of cells with nuclear staining were quantified by setting a colour threshold for brown (positive nuclei) and blue (negative nuclei) staining, which was set for every slide analyzed. Slides were reviewed and all the tumour area was evaluated to a 40 field.

2.4. *Sample preparation and analysis of cell cycle proliferating genes*

Formalin-fixed and paraffin-embedded biopsy tumour blocks underwent pathologic evaluation. The original diagnostic haematoxylin and eosin-stained (HE) tissue sections from each block were evaluated for tumour content. The tumour area was identified, measured (length milimeters), and circled. Additionally, 12 sections of tissue were cut: 2 for HE (#1 and #12, 3–5-μm section) and 10 (#2–#12, 10-μm section) unstained for subsequent RNA isolation.

Following pathologist's instructions, some selected tumour regions were removed from unstained slides by macrodissection. The tumour region was dissected directly into a centrifuge tube and the paraffin removed using xylene and washed with ethanol.

Proteinase K digestion at 55 °C was used overnight to treat samples. Total RNA was extracted using miRNeasy (Qiagen, Valencia, CA) as described by the manufacturer (with the exception of the extended proteinase K digestion). Isolated total RNA was treated with DNase I (Sigma, St. Louis, MO) before complementary DNA (cDNA) synthesis. Total RNA was converted into single-strand cDNA using high-capacity cDNA. Archive Kit (Applied Biosystems, Foster City, CA) as described by the manufacturer. The cDNA was preamplified with a pooled reaction containing 31 CCP and 15 housekeeping gene TaqMan assays before measuring expression levels. Preamplification reaction conditions were 95 °C for 10 min, 95 °C for 15 s, and 60 °C for 14 cycles and dilute 1:20 using 1× Tris-EDTA buffer before loading on Taqman Low Density Arrays (Applied Biosystems) to measure gene expression. All samples were run in triplicate.

2.5. *CCP score calculation*

The CCP score was calculated from the expression data of 31 CCP genes normalized by 15 housekeeping genes as previously described.[4] Neither CCP scores were rejected, nor standard deviation of CCP scores (triplicate > 0.5) were observed.

In order to adjust (correlate, compare) the CCP score, and tumour aggressiveness based on clinical high-risk classification[11] different intervals of aggressiveness were identified: *considerably less aggressive* (CCP score less than −2.0; level −2), *less aggressive* (CCP score between −2.0 and −1.0; level −1), *consistent* (CCP score between −1.0 and 0.0; level 0), *more aggressive* (CCP score between 0.0 and 1.0; level 1), *and considerably more aggressive* (CCP score more than 1.0; level 2). The threshold between intervals is one unit of the CCP score, with the *consistent* interval centred at the median CCP score. Patients with a CCP score more than 0.0 indicated that this cancer was more aggressive than the average cancer in the D'Amico high-risk category.[9,11]

CCP score and 10-year prostate DSM risk were also correlated based on clinic-pathologic features, such as patient age, PSA prior to biopsy, % of positive cores, Gleason score, and clinical T stage, as was published in a previous trial.[12]

2.6. *Endpoints*

BF was defined as reaching a post-RT PSA of nadir + 2 ng/ml (Phoenix criteria, 7 patients). DSM was defined as death resulting from prostate cancer showing progression or unknown causes with previously documented clinical or biochemical recurrence. Overall survival (OS) was defined as death resulting from any cause. Distant metastasis (DM) was defined as radiographic or clinical evidence of spread. Imaging studies to screen for DM at follow-up were only ordered when deemed clinically indicated by treating physicians.

2.7. Statistical analysis

All time events were measured from the date of radiation treatment to the date of their occurrence or last follow-up. The majority of the analysis is based on 10-year censoring to address the observed time dependence of the hazard ratio (HR) for CCP and Ki-67 and BF and DSM.

All pre-treatment characteristics recorded were age at diagnosis, baseline PSA level, clinical stage, Gleason score grouped into 3 categories: <7, 7, and >7, concurrent hormone use, D'Amico risk classification,[11] radiation dose, percent positive cores, total percent cores, and perineural invasion. All CCP scores and Ki-67 expression were assigned before unmasking the clinical and outcome data.

The Kaplan–Meier method[13] was used to estimate yearly survival rates for OS, DSM, DM and BF. A Cox proportional hazards model was used for univariate and multivariate analyses to assess the added prognostic information of the CCP score and Ki-67 on risk failure. Hazard ratio was calculated for each variable factor without adjustment because the total number of patients (33) did not allow to include more than one variable into the model. The association of the different variables analyzed with BF, OS, and DSM (age, tumour, PSA, and Gleason score, D' Amico risk group,[11] RT dose, nadir PSA post-RT, time to nadir PSA post-RT, interval to relapse alter treatment, ADT) was evaluated with the Chi-square test and their association with outcome measures was determined with the log-rank test and the Cox regression method. Two-sided p values of less than 0.05 were considered statistically significant.

Statistical inference was conducted within the STATA (version 13.0) software environments.

2.8. Follow-up

Patients were followed-up by the Radiation Oncology staff every 6 months for the first 4 years, and on a yearly basis thereafter.

3. Results

The cohort included 33 pts with high-risk adenocarcinoma of the prostate. The clinical characteristics are shown in Table 1.

With a median follow-up of 8.8 years (IQR 7.3, 8.8 year; range, 2.4–10.6 year), the 10-year actuarial free-from DSM, OS, and BF was 87%, 78% and 74%, respectively.

A total of 7 (21%) pts had BF (median 44 months; IQR 15, 63 months). Five pts had BF within the first 5-year after RT. Three (9%) pts had DSM (median 8.2 year; IQR 7.2, 8.8 year).

CCP risk of tumour aggressiveness based on clinical high-risk classification (11) showed that 85% of pts were classified as *more aggressive* (23 pts, 70%; level 1) or *considerably more aggressive* (5 pts, 15%; level 2).

There was a positive interaction (Pearson correlation) between the CCP score and Ki-67, and between the CCP score and Gleason, with a borderline statistical significance (Fig. 1).

Both the manual scoring method and image-analysis determination (ACIS) for Ki-67 were used. Correlations with outcomes were stronger for the manual method than for image-analysis determination; therefore, only, the manual Ki-67 results are reported.

The median Ki-67 was 6.8. We defined the Ki-67 cutpoint on the area under ROC curve (AUC) for BF. Optimizing sensibility and specificity, the proposal cutpoint for Ki-67 is $\geq$15.08 (AUC = 0.5934, $p = 0.043$, 95% CI 0.32–0.87).

On univariate analysis, Ki-67 > 15% was associated with BF ($p = 0.004$, OR 8.98, 95% CI 1.6–50.3), OS ($p = 0.023$, OR 8.01, 95% CI 1.3–48.2), and DSM ($p = 0.01$, OR 23.1, 95% CI 2.09–25.6). On Cox multivariate analysis, KI-67 > 15% was associated with a trend BF ($p = 0.063$).

On uni- and multivariate analysis, we associated the CCP score by levels (level 1 vs. level 2) and CCP as a continuous variable (median CCP < 0.8 vs. >0.8) with BF, OS and DSM at 10-year. Even though we observed a worse tendency in BF ($p = 0.76$ vs. 0.71), OS ($p = 0.83$ vs. 0.73), and DSM ($p = 0.94$ vs. 0.78) for patients with the CCP 2 score or CCP > 0.8, it was not statistical significant. Fig. 2 illustrates the CCP score and DSM at 10 years.

4. Discussion

To our knowledge this study represents the first example of a prognostic molecular biomarkers (CCP genes and Ki-67), from tissue obtained from diagnostic needle biopsies, for high-risk prostate cancer patients primarily treated with radiation therapy.

High-risk and very high-risk of PCa pts has the most unfavourable clinical outcome after radiation therapy at 10 years. The ability to predict clinical outcome is essential to implement optimal strategies to treat these patients.[14,15,20,21]

In this study, we demonstrate the CCP and Ki-67 derived from the diagnostic biopsy have prognostic clinical information on aggressiveness, BF, OS, and DSM after primary RT. These results are comparable with previous reports on Ki-67 and CCP genes expression studied separately.[3–6,8–10]

In the present study, the CCP score classified 85% (28/33) of pts more aggressive than did a clinical risk stratification.[11] Expression of CCP genes is higher in actively growing cells and, indirectly, we are measuring the growth rate and inherent aggressiveness of the tumour. That will affect the clinical outcome. Several authors,[4,8–10] in a cohort of PCa pts treated with RTU, surgery or radiation therapy have previously demonstrated a robust correlation between a high CCP score and aggressiveness. The prognostic information provided by the CCP score provides better risk discrimination across the amount of clinical risk. Freedland[8] evaluated the prognostic utility of the CCP score in a cohort of 141 localized PCa pts treated with RT. In this study, the CCP score was mostly independent of other clinical variables.

High CCP score is a predictor of BF and mortality.[8,9] BF after primary RT in localized PCa occurs frequently after 5 years from treatment. Early relapse (<5-year) after RT is often associated with poor outcome.[16] In our study, 6 of 7 pts with BF after RT were classified as *more aggressive* or *considerably more aggressive* by the CCP score. Consistent with this evidence, we observed that 5 of 7 pts had BF before 5 years after RT, and 3/5 BF relapsing very early (less than 2 years after RT) died. The median follow-up of our series was 8.8 years with OS of 78% at 10 years.

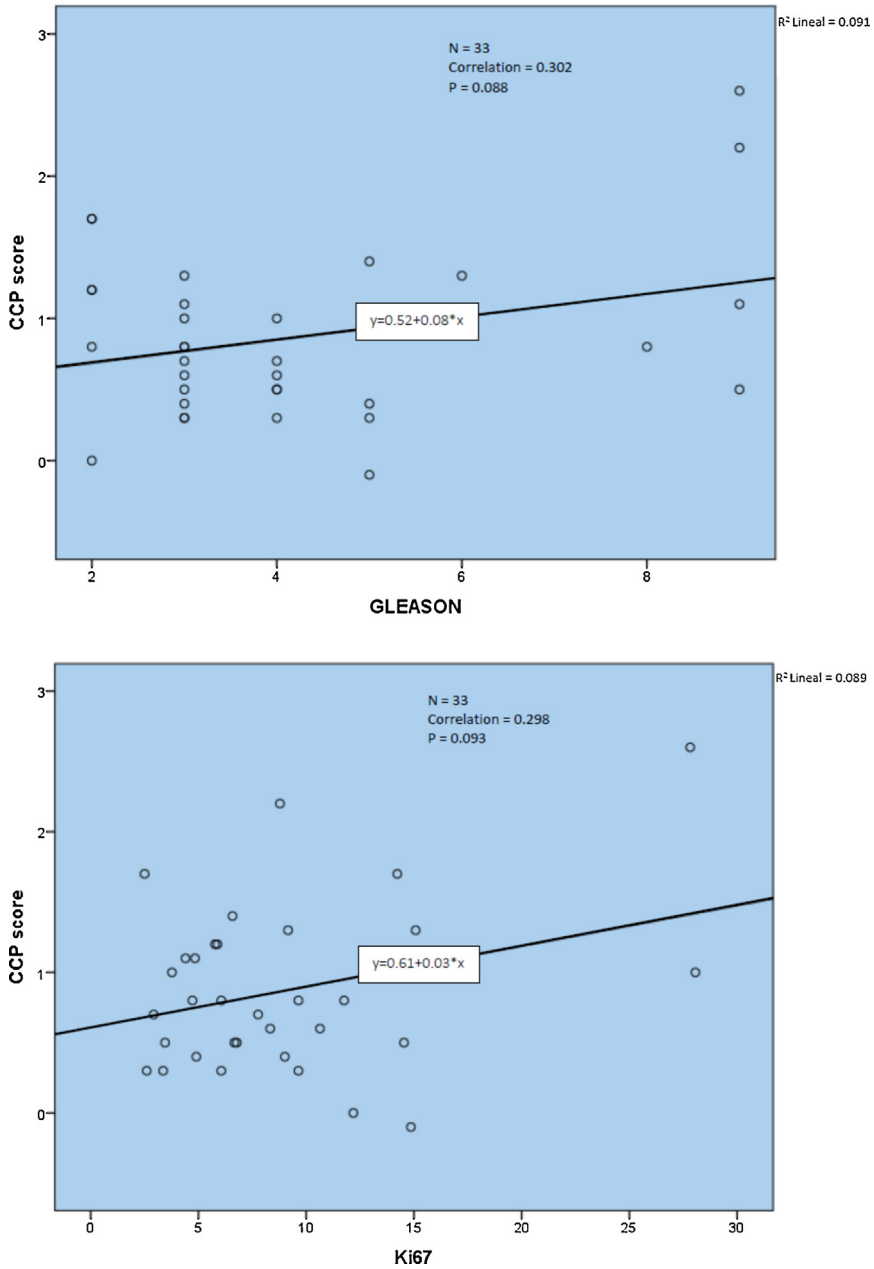

Fig. 1 – Pearson correlation between CCP score and Gleason and Ki-67 score.

Even the median follow-up reported by Freedland[8] was 4.8 years, 19 (13%) pts had BF, and 9 of them had early relapsed (first 5 years after RT). The author argued that the CCP score was not only a strong predictor of BF but also of early treatment failures ($p = 0.0063$). Also, 6 pts died from prostate cancer within 10 years. The CCP score was associated with DSM ($p = 0.013$).

We found a trend towards a larger correlation effect of the CCP score and Ki-67 and between the CCP score and Gleason score > 7 (Fig. 1). Although this trend was not significant and in contrast to previous controversial results,[4,8,9] if confirmed, it suggests that the CCP score may help stratify better high-risk localized prostate cancers into "favourable high-risk" and "unfavourable high-risk" groups, as it is been suggested from clinical factors in a previous study.[17] In addition to this, early failures (before 5 years) are more indicative of very aggressive disease and, therefore, clinically relevant due to higher possibility of these pts for disease specific mortality.[9,14] If true, then a high CCP score at diagnosis predicts micrometastatic disease, and these events would indicate the need for more aggressive treatment initially (i.e. higher doses of RT and ADT and novel agents). However, even with the longer follow-up of this series, and a tendency of poor outcome of pts with a high CCP score, as noted, given the small sample size, small number of treatment failures, we did not find any statistical significance of the CCP score neither with BF nor DSM. To have a definitive conclusion regarding a higher CCP score and time dependency, additional studies will be required.

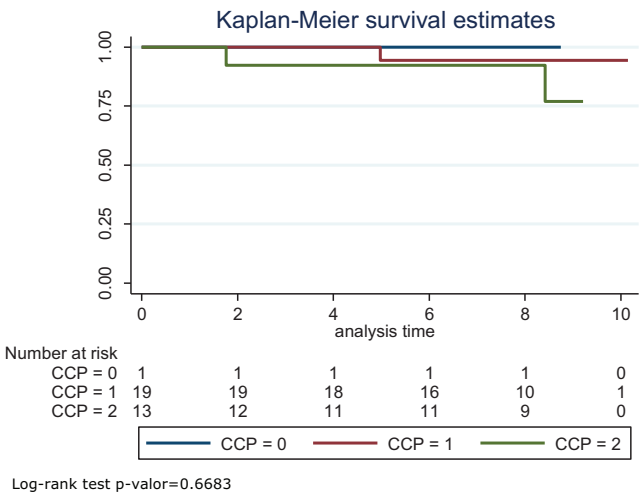

Fig. 2 – Kaplan–Meier of CCP score and DSM at 10-year survival. Log-rank test *p*-value = 0.6683.

In contemporary prostate cancer studies with different management approaches, Ki-67 has been found to be an independent predictor of biochemical failure, distant metastasis and cause-specific mortality in prostate cancer pts treated with RT.[3,6,7,14,18,19]

In this study, derived from quadrant analysis, a Ki-67 > 15.08% labelling index cutpoint was strongly and independently predictive of BF, OS, and DSM on univariate analysis, with a trend of BF on multivariate analysis. These results are consistent with previous RTOG trials on Ki-67 and BF, DM, and DSM, not only in pts with low-intermediate PCa (3), but also in pts with high-risk[6,7] PCa treated with RT. A foremost potential concern in the present study would be the small number of pts included (33 pts), and the limitations to make strong conclusions.

The results of the present study are far from conclusive due to the small number patients. Despite the retrospective nature of the study, all specimens included for prognostic correlation of the CCP score and Ki-67 were from high-risk PCa pts treated with the same radiation technique and doses with a longer follow-up, and this may minimize a potential for selection bias, and may not preclude the identification of some patients and treatment-related factors that might influence the outcomes. Nonetheless, even with these limitations, we still demonstrated consistent results according to other prior studies. In addition, we identified some potential correlation of the CCP score and Ki-67 as a molecular predicting factor for aggressiveness, BF and DSM in high-risk PCa pts treated with RT. However, we must be cautious to further investigate these molecular predicting factors of outcome in prospective trials.

5. Conclusions

High CCP score and Ki-67 from formalin-fixed, paraffin-embedded prostate core needle biopsy tissues have strong correlation to predict aggressiveness, BF and DSM in high-risk PCa pts treated with RT.

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study no formal consent is required.

Author's contributions

IH participated in the sequence alignment and drafted the manuscript. DP carried out the immunoassays. AB participated in the design of the study and performed the statistical analysis. MA, OA, MA, KP, FR, PG, MG conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Conflict of interest

This study was supported by a partial unrestricted collaboration from Myriad Genetics. Myriad Genetics carried out the molecular genetic studies. Myriad Genetics did not influence on any part of written manuscript.

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