Original Article

Ki-67, topoisomerase IIα and miR-221 have a limited prostate cancer risk stratification ability on a medium-term follow-up: results of a high-risk radical prostatectomy cohort

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Background: Currently, no biomarkers are able to differentiate lethal from relatively indolent prostate cancer (PCa) within high-risk diseases. Nonetheless, several molecules are under investigation. Amongst them, topoisomerase-II-alpha (*TOPIIA*), *Ki67* and *miR-221* showed promising results. Our aim was to investigate their prognostic role in the context of biochemical recurrence (BCR), clinical recurrence (CR) and PCa-related death (PcD).

Methods: We included 64 consecutive cM0 high-risk PCa [prostate specific antigen (PSA) >20 ng/mL or Gleason Score (GS) >7 or cT >2] undergoing radical prostatectomy (RP). Changes in miR-221 expression and alternative splicing were determined using microarrays. Immunohistochemical determination of Ki67 and TOPIIa were performed using monoclonal antibody MIB-1 and 3F6 respectively. Cox proportional-hazards regression models were used to predict BCR and CR as multivariate analysis. BCR and CR were defined as three consecutive rises in PSA and PSA >0.2 ng/mL and histologically-proven local recurrence or imaging positive for distant metastasis respectively.

Results: We included 64 men. Mean pre-operative PSA was 26.53 (range, 1.3–135); all GSs were ≥7 and pT was ≥ T3 in 78.13%. Positive margins and lymph-nodes were present in 42.19% and 32.81% respectively. At a mean follow-up of 5.7 years (range, 1.8–12.5), 42.18% experienced BCR (n=27), 29.68% CR (n=19) and 7.81% PcD (n=5). On univariate analysis positive nodes (<0.01), seminal vesicle invasion (0.02) and *miR-221* downregulation (P=0.03), but not *Ki67* and *TOPIIA* (both P>0.5) were associated with BCR whereas only PSA (P<0.01), seminal vesicle invasion (P<0.01) and positive nodes (both P<0.01) were linked to CR. No parameters predicted PcD (all P>0.05) or BCR and CR on multivariate analysis (all P>0.05 - miR-221 HR 0.776; 95% CI: 0.503–1.196 for BCR and HR 0.673; 95% CI: 0.412–1.099 for CR). Limitation of the study include its small sample size and limited follow-up.

Conclusions: *TOPIIA*, *Ki-67* and *miR-221* may not predict BCR, CR or PcD in high-risk PCa patients who underwent RP at a medium-term follow-up. Longer follow-up and larger cohorts are needed to confirm our findings.

Keywords: Risk stratification; radical prostatectomy (RP); miR-221; Ki-67; topoisomerase IIα

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Introduction

Prostate cancer (PCa) remains the commonest non-skin cancer solid neoplasm amongst men, with the majority of diseases being discovered at an early indolent stage (1,2). However, due to its high incidence, it represents the second cause of male cancer-related deaths, claiming the need of a prognostic marker able to better identify aggressive diseases, potentially lethal, especially if undertreated (1).

Currently amongst the top four causes of cancer related deaths, PCa is the only neoplasm lacking such a tool, unlike breast, colorectal and non-small cell lung cancer, for which targeted therapies in selected subgroups of patients led to significant survival improvements (1,3-5).

In the intrinsic heterogeneity of PCa, high risk cancers are indeed the most relevant subgroup in which a potential biomarker may be tested to verify its predicting ability. To date, PSA, tumour stage and GS remain the major instruments to predict the risk of recurrence after radical prostatectomy (RP). Despite their proved ability to differentiate low-, intermediate- and high-risk diseases, no tools exist to further define aggressive diseases. Risk stratification is currently based on clinical parameters only, leading to a clinically defined uniform risk class in which, however, risk of dying for PCa competing causes after radical treatment can significantly differ from one case to another, with reported 10-year rates ranging from 9% up to 61% (6,7): this claims an absolute need of accounting for inter-individual outcomes to enhance disease-free and cancer-specific survival. With this aim, several biomarkers are currently under investigation. Hence, prognostic markers able to predict either the risk of biochemical recurrence (BCR) and clinical recurrence (CR) or the risk of PCa-related death (PcD) would revolutionize the treatment allocation pathway, allowing more precise risk-based discrimination.

MicroRNAs (miRNA) are non-coding RNA molecules able to regulate gene expression by hybridizing with target mRNA complementary sequences. Their alteration has been shown to occur in both *in vivo* and *in vitro* cancerous cells, making them ideal cancer biomarkers and/or therapeutic targets (8,9). Above all, *miR-221* has been suggested as a potential PCa biomarker, being inversely associated with the risk of PCa recurrence (8,9).

Many microRNAs have been studied concerning PCa, including *miR-21* (8,10), *miR-141* (8), *miR-205* (11), *miR-214* (11), *miR-222* (10,12,13) and others. Nonetheless, bioavailability is not always promising for their introduction in clinical practice and evidence is sometimes contrasting. Amongst them, *miR-221* shows the most promising evidence, based on PCa cells *in vitro* studies which suggest its involvement in several molecular pathways such as androgen-dependent cell growth and development of CRPC phenotype (12,14,15), PCa migration and invasion (16,17), and inhibition of several cyclin-dependent kinases complexes, including those inducing apoptosis (13,18). Furthermore, *miR-221* levels can be obtained from blood, prostatic tissue and prostatic secretion, thus providing easily available samples (19). Disagreement characterises the early clinical evidence. Three studies found no association amongst *miR-221* downregulation and either BCR or CR (10,20), whilst four demonstrated significant correlations (8,9,16,17).

Amongst molecules that have been incorporated in models predicting the risk of PcD, *nuclear-based protein Ki-67*, expressed in cells undergoing proliferation as an expression of DNA synthesis, and *DNA binding enzyme topoisomerase-2α* (*TOPIIA*) also appear as promising tools (21,22). The former yielded good correlation with BCR and enhanced D'Amico risk stratification predicting ability of post-RP outcomes (21) and the latter correlated with risk of systemic PCa progression (22).

In the present study we report the results concerning the analysis of *miR-221*, and, given previous studies from others and our group reporting promising results, *Ki-67* and *TOPIIA* prognostic role in men with high risk PCa who underwent RP. We present the following article in accordance with the REMARK reporting checklist (available at https://tau.amegroups.com/article/view/10.21037/tau-21-628/rc) (23).

Methods

Participants

After receiving institutional review and ethics board approval, we retrospectively collected data of 64 consecutive patients who underwent RP with extended lymph node dissection

for high risk PCa at San Giovanni Battista Hospital, Turin, Italy, between 2003 and 2011. Patients were followed up with PSA measurements every three months for the first year, every six months in the following four years and yearly thereafter. Clinical visits were performed every six months for the first two years and then yearly. In case of PSA rise and/or suspicion of recurrence cases were discussed at a multidisciplinary meeting including Urologists, Oncologists, Radiologists, Radiotherapists and Nuclear Medicine Specialists and subsequently managed (imaging/staging) and/or treated (surveillance/adjuvant/salvage treatments) depending on the panel's recommendation, in line with the EAU guidelines for prostate cancer.

Inclusion and exclusion criteria

Inclusion criteria were presence of high risk PCa, confirmed by the RP specimen and defined according to D'Amico Criteria [Gleason Score (GS) ≥8 and/or prostate specific antigen (PSA) >20 ng/mL and/or cT ≥3a]. Neo-adjuvant and adjuvant hormonal therapy as well as adjuvant radiotherapy were not considered as exclusion criteria.

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the institutional ethics board of San Giovanni Battista Hospital (No. 112/106/70/2017) and individual consent for this retrospective analysis was waived.

Measures

All men received a negative pre-operative clinical staging including PSA, digital rectal examination, abdominal computed tomography (CT) and bone scans. Other recorded clinical variables included age, height, weight, BMI, follow-up PSA measurements, number and percentage of positive nodes (confirmed by histological analysis), cTNM, Charlson Comorbidity Index and American Society of Anaesthesiologists (ASA) score.

Outcomes

BCR and CR were defined as three consecutive rises in PSA and PSA being >0.2 ng/mL after RP and histologically proven local recurrence or bone or CT scan distant metastasis respectively. PcDs were verified by phone interviews.

Our primary endpoint was to evaluate *miR-221*, *Ki-67* and *TOPIIA* their prognostic role in the context of BCR, CR and PcD by comparing the expression of each marker in those experiencing BCR and/or CR and/or PcD versus those who did not.

Pathological processing and markers analysis

Pathological macro-dissection was performed according to the Stanford protocol. After initial diagnostic evaluation all RP specimens were reviewed by a senior uro-pathologist and index lesions (defined as the largest cancer focus and/or focus with the highest GS) were contoured. No immunostaining was performed at this stage. After pathological examination, paraffin-embedded tissue was analysed for *miR-221*, *Ki-67* and *TOPIIA* levels.

RNA extraction and miR-221 analysis

Changes in miRNA expression levels, alternative splicing, or expression of mRNAs were determined using microarrays (e.g., GeneChip® Exon Arrays, Affymetrix) and quantitative RT-PCR methods.

Total RNA Extraction Kit (Applied Biosystems) was used to extract totalRNA as described previously (9). Quality and concentration of extracted RNA was determined with a BioAnalyzer (Agilent). cDNA was synthesized from total RNA with stem-loop reverse transcription primers for miR-221 according to the TaqMan MicroRNA Assay protocol. TaqMan microRNA assay kits and an Applied Biosystems 7900HT system were used for quantification of microRNA in tissue samples according to the manifacturer's protocol (Applied Biosystems). For normalization and subsequent calculation of relative miR expression we used *miR-151-3p* expression and the comparative DCt-method. Mean Ct was determined from triplicate PCRs.

Immunohistochemical analysis: Ki-67 and TOPIIA determination

Immunostaining was performed as previously described (21,22). The monoclonal antibody MIB-1 (Immunotech, Marseilles, France; 1:400 dilution) using a standard avidin-biotin complex method to determine cells positivity was used for *Ki-67* staining.

An automatic stainer (BioTek, Ventana Medical Systems, Tucson, AZ, USA) was used for slides processing. Hematoxylin dilution was used as nuclear counterstain. MIB-1 percentage of positive nuclear area (MIB-1 index) was obtained with the Quantitative Proliferation Index program of the CAS 200 image analyser. Total optical density of nuclei expressing the antigen that reacts with MIB-1 (the brown chromogen diaminobenzidine) was

Table 1 Baseline patient characteristics

Variable	n	Median [IQR]
PSA pre-op (ng/mL)	64	19.5 [1.3–135.0]
Age (years)	64	69 [53–79]
Height (cm)	56	171.5 [157–185]
Weight (Kg)	56	75.5 [54–100]
BMI	56	24.8 [18.7–36.3]
ASA Score	56	2 [1–4]
Charlson Score	63	1 [0–7]

PSA, prostate specific antigen; BMI, body mass index; ASA, American Society of Anaesthesiologists.

divided by the total optical density of all measured nuclear images.

Monoclonal antibodies 3F6 (Novacastra, Benton Lane, UK, 1:100 dilution) was used for *TOPIIA* immunostaining. Dako Advance polymer-based detection system (Dako, Carpenteria, CA, USA) was used for stain performance. Slide Scanner and Immunostaining Score Software (Bacus Laboratories, Inc., Lombard, IL, USA) were used for slides scanning and to obtain measurements of the 3F6 (total and immune-reactive nuclear area of the invasive tumour component within the index lesions).

MIB-1 and 3F6 immunostaining were expressed as the percentage of invasive tumour total nuclear area that stained positively [labeling index (LI)]. After digital imaging analysis all slides were visually reassessed by dedicated cytotechnologists in relation to the MIB-1 and 3F6 LIs to guarantee correct quantification. *Ki-67* and *TOPIIA* were defined as the percentage of PCa cells nuclear area displaying MIB-1 36F staining, irrespectively of their intensity.

Statistical analysis

Different distributions of all independent prognostic variables recorded were compared according to presence or absence of BCR, CR and PcD using Wilcoxon non-parametric test for dichotomic and Mann Whitney U-test for continuous variables.

For univariate analysis Kaplan-Meyer curves were plotted for each independent variable (age, PSA, GS, positive and percentage of positive nodes, surgical margins status, height, weight, BMI, Charlson Score, *Ki-67*, *topoisomerase II* and *miR-221*) according to BCR, CR and PcD respectively. Cox proportional-hazards regression models to predict Biochemical and clinical recurrence (CR) as multivariate analysis. Statistics were conducted using SAS ver. 9.4 for Windows (SAS Institute, Cary, NC, USA) software package. P values of ≤0.05 were considered significant.

Results

Baseline clinical characteristics are shown in *Table 1*, whereas *Table 2* reports baseline pathological features. Mean pre-operative PSA and age were 26.5 (range, 1.3–135) and 68 (range, 53–79), with clinical stage being organ confined in 82.3% (n=51) and biopsy Gleason Score (bGS) being ≥7 in 92.1% (n=58). RP specimen pathological features were extracapsular extension (≥ pT3) in 78.1% (n=50) (pT4=3.1%, n=2 and SVI =39.1%, n=25), all being GS ≥7. Rates of positive surgical margins and lymph-nodes were 42.2% (n=27) and 32.8% (n=21) respectively. Additional PCa treatments including adjuvant and neo-adjuvant ADT and radiotherapy are displayed in *Table 3*. At a mean follow-up of 5.7 years (range, 1.8–12.5), 42.2% of the cohort (n=27) experienced BCR, and 29.7% had CR (n=19), of whom 7 locally and 12 in distant sites; overall death was 9.4% (n=6), 5 men died because of PCa (PcD =7.8%) and 1 for other causes. Kaplan-Meyer curves for BCR and CF are shown in *Figure 1*. On univariate analysis (*Table 4*) number of positive nodes (<0.01), positive nodes status (P<0.01), seminal vesicle invasion (0.02) and *miR-221* downregulation (P=0.03) were significant predictors of BCR. However, only PSA (P<0.01), seminal vesicle invasion (P<0.01) and positive nodes number and status (both P<0.01) were able to predict CR. *Ki-67* and *TOPIIA* levels were not associated with enhanced risk of BCR and/or CR (all P>0.5). Amongst investigated parameters, none was able to predict PcD (all P>0.05). On multivariate analysis for BCR and CR (Table S1), none of the investigated variables was able to independently predict PCa outcomes (all P>0.05).

Discussion

In the current study we evaluated *miR-221*, *Ki-67* and *TOPIIA* prognostic ability in a high risk PCa cohort with a mean follow-up of 5.7 years. *miR-221* was able to predict BCR but not CR and PcD, whereas no correlation of the latter three with *Ki-67* and *TOPIIA* levels were found.

To date, PSA, tumour stage and GS remain the major instruments to predict the risk of recurrence after RP. Despite their proved ability to differentiate low,

Table 2 Pathological characteristics of the study cohort

Variable	n (%)
Clinical stage	62 (96.9)
T1	15 (24.19)
T2	36 (58.06)
T3	11 (17.74)
Pathological stage	64 (100.0)
T1	3 (4.69)
T2	11 (17.19)
T3	48 (75.0)
T4	2 (3.13)
Biopsy Gleason Score	63 (98.4)
≤6	5 (7.9)
7	26 (41.3)
8	17 (27.0)
9	15 (23.8)
10	0 (0.0)
Pathological Gleason Score	64 (100.0)
≤6	0 (0.0)
7	20 (31.7)
8	22 (34.9)
9	22 (34.9)
10	0 (0.0)
Seminal vesicle invasion	64 (100.0)
Yes	25 (39.06)
No	39 (60.94)
Surgical margins	64 (100.0)
Positive	27 (42.19)
Negative	37 (57.81)
Pathological lymph nodes	64 (100.0)
Positive	21 (32.81)
Negative	43 (67.19)
Removed (median, range)	18 (0–62)
Positive (median, range)	0 (0–31)

Table 3 Adjuvant, neo-adjuvant and salvage therapies in the study cohort

Variable	n (%)
Neo-adjuvant ADT	64 (100.0)
Yes	11 (17.19)
No	53 (82.81)
Adjuvant-treatment	64 (100.0)
ADT	
Yes	24 (37.5)
No	40 (62.5)
Radiotherapy	
Yes	23 (35.94)
No	41 (64.06)

ADT, androgen deprivation therapy.

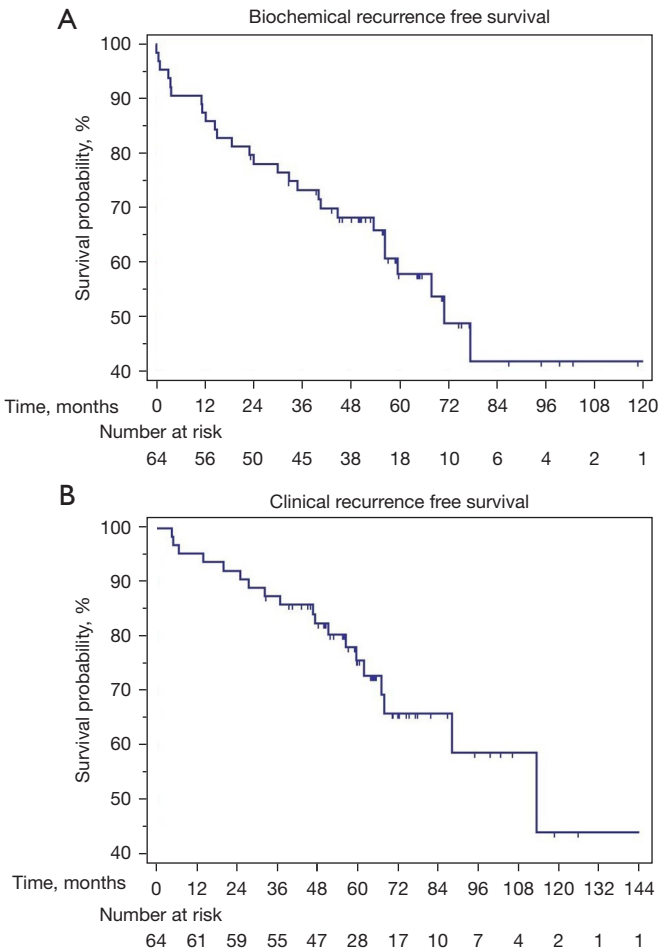

Figure 1 Kaplan-Meyer curves for biochemical recurrence (A) and clinical recurrence (B) free survival.

Table 4 Univariate analysis for biochemical and clinical recurrence

Variable	Biochemical recurrence, mean (SD)/n (%)			Clinical recurrence, mean (SD)/n (%)		
	No	Yes	P	No	Yes	P
Clinical features						
PSA (ng/mL)	20.98 (25.4)	34.14 (29.5)	0.06	17.59 (14.6)	47.69 (38.9)	<0.01
cT≥3	4 (6.2)	2 (3.2)	0.11	5 (8.1)	1 (1.6)	0.42
Biopsy GS ≥8	17 (27.0)	15 (23.8)	0.51	20 (31.7)	12 (19.0)	0.20
Age (years)	66.81 (6.6)	69.59 (6.1)	0.09	67.2 (6.5)	69.84 (6.2)	0.14
Height (cm)	171.78 (6.4)	171.12 (6.8)	0.72	170.74 (6.6)	173.11 (6.4)	0.21
Weight (kg)	76.84 (9.8)	76.12 (11.4)	0.80	76.53 (10.5)	76.56 (10.9)	0.99
ASA score	2.37 (0.7)	2.208 (0.6)	0.43	2.21 (0.7)	2.5 (0.6)	0.14
BMI (Kg/m^2)	26.11 (3.7)	25.98 (3.6)	0.68	26.3 (3.7)	25.53 (3.4)	0.84
Charlson	1.52 (1.6)	1.78 (2.1)	0.86	1.43 (1.6)	2.10 (2.3)	0.32
Biomarkers						
Δ miR-221	2.62 (1.3)	1.69 (1.6)	0.03	2.39 (1.4)	1.92 (1.7)	0.31
TOPIIA staining (%)	0.92 (1.2)	1.23 (1.8)	0.62	1.03 (1.4)	1.13 (1.8)	0.76
Ki-67 staining (%)	2.47 (2.5)	3.50 (4.8)	0.99	3.04 (3.7)	2.58 (3.6)	0.33
Radical prostatectomy pathology						
GS ≥8	24 (37.5)	20 (31.2)	0.43	29 (45.3)	15 (23.4)	0.25
Stage ≥ pT3	30 (46.9)	21 (32.8)	0.75	34 (53.1)	17 (26.6)	0.21
SVI	10 (15.6)	15 (23.4)	0.02	12 (18.75)	13 (20.3)	<0.01
Positive margins	12 (18.7)	15 (23.4)	0.06	16 (25.0)	11 (17.2)	0.10
Lymph node status						
Removed	21.27 (14.1)	19.04 (11.1)	0.73	19.16 (12.3)	22.5 (13.7)	0.47
Positive	1.43 (5.8)	3.04 (6.6)	<0.01	0.22 (0.5)	6.0 (9.7)	<0.01
pN+ patients	5 (7.8)	16 (25.0)	<0.01	8 (12.5)	13 (20.3)	<0.01
Additional treatments						
Neoadjuvant ADT	4 (6.25)	7 (10.94)	0.11	6 (9.4)	5 (7.8)	0.2085
Adjuvant ADT	7 (10.9)	17 (26.6)	<0.01	10 (15.6)	14 (21.9)	<0.01
Adjuvant RT	11 (17.9)	12 (18.7)	0.23	13 (20.3)	10 (15.6)	0.07
Adjuvant ADT and/or RT	15 (23.4)	17 (26.6)	0.08	17 (26.6)	15 (23.4)	<0.01
Salvage treatment	4 (6.2)	15 (23.4)	<0.01	8 (12.5)	11 (17.2)	<0.01

PSA, prostate specific antigen; GS, Gleason Score; ASA, American Society of Anaesthesiologists; BMI, body mass index; SVI, Seminal vesicle invasion; RT, radiotherapy; ADT, androgen deprivation therapy.

intermediate and high-risk diseases, no tools exist to further define aggressive PCa. Amongst those with the greatest malignant potential, we are not able to predict which patients will remain PCa-free and which patients will develop recurrence after primary curative treatment, possibly benefitting from adjuvant or more aggressive first line approaches. As shown in our cohort, with 42.18% BCR, 29.68% CR and 7.81% PcD, high risk PCa yields

significant progression rates. Reported 5 years BCR and PcD range from 37 up to 65% and from 1 up to 7% respectively (24): this claims an absolute need of accounting for inter-individual outcomes to enhance disease-free and cancer specific survival. With this aim, several biomarkers are currently under investigation.

Ki-67 and *TOPIIA* have been assessed in several malignancies, including breast (25-27), lung (28,29) and other major neoplasms (30-33). *TOPIIA* is involved with cell proliferation, with a well-characterized role in the DNA repair. Increased *TOPIIA* expression has been associated with enhanced risk of PCa progression through an increase in androgen-related signalling (34). Conversely, whilst being associated with cell proliferation, the biological function of *Ki-67* remains unknown.

Many series highlighted *TOPIIA* high levels correlate with GS (35-40), preoperative PSA (37,40) and surgical stage (36); a few of these works also proved its independent ability in predicting BCR (22,35,37), and, in two cases, its association with a decreased survival after PCa radical treatment (22,35).

Ki-67 was recently validated as an independent predictor of BCR in a large multi-centre cohort of 3,123 men (HR =1.19; P=0.019) (41). Smaller studies proved its ability in predicting PcD as well (21,42). A work from the Mayo Clinic found *Ki-67* as an independent predictor of PcD; each 1% increase in *Ki-67* expression related to a 12% growth in cancer-specific death (P<0.001) (21).

As in the current study, Karnes and colleagues tested both *TOPIIA* and *Ki-67* in prognostic models (22). However, in their work, the staining of *TOPIIA* and *Ki-67* were significantly different for PCa experiencing systemic progression after RP compared to those who did not (P<0.001).

Further sub-analysis assessed prognostic ability in relation to *ERG*, an oncogenic protein which binds other proteins to modulate cytoskeleton organization, cell migration and protein degradation. *ERG* gene rearrangement with common recurrent transmembrane protease serine 2 (TMPRSS2) causes its overexpression. This seems to be an early step of PCa genesis and, although its role remains unclear, it has been associated with higher GS, stage and PCa aggressiveness (22,43).

In men without *ERG* translocation, *TOPIIA* had a significantly better prognostic ability than *Ki-67*. On the contrary, when *ERG* translocation was present, *TOPIIA* and *Ki-67* had no predictive value, suggesting their prognostic role may vary depending on *ERG* status (22). This has been recently confirmed in a large RP cohort (43). Although GS, pre-op PSA and positive lymph nodes were associated with a higher risk of biochemical and/or CR, in our study, no significant correlations with BCR, CF and PcD were found for either *Ki-67* or *TOPIIA*. Indeed, the *ERG* status may account as an important influencing factor. In this sense the relatively small number of our validation cohort may even have been reduced by the presence of *ERG(+)* men, on whom *Ki-67* and *TOPIIA* do not show any significant predictive ability. Unfortunately, we did not assess *ERG* translocation and no concrete statements can be made regarding this hypothesis. It is our opinion that future studies on *Ki-67* and *TOPIIA* need to consider *ERG* status to account for possible biases deriving from its role, and to further clarify its mechanism of action. Low cost and rapid assessment, especially for *Ki-67*, are certainly considerable advantages that may derive from their large-scale use if prognostic ability is confirmed by future research. A retrospective analysis of *ERG* translocation has been planned in our cohort and will likely shed light on our current results.

Differently from *Ki-67* and *TOPIIA*, miRNA are relatively younger molecules, whose association with cancer was first suggested in 2002 for chronic lymphocytic leukemia (44). Many microRNAs have been studied concerning PCa, including *miR-21* (8,10), *miR-141* (8), *miR-205* (11), *miR-214* (11), *miR-222* (10,12,13) and others. Amongst them, *miR-221* shows the most promising evidence, based on PCa cells *in vitro* studies which suggest its involvement in several molecular pathways such as androgen-dependent cell growth and development of CRPC phenotype (12,14,15), PCa migration and invasion (16,17), and inhibition of several cyclin-dependent kinases complexes, including those inducing apoptosis (13,18). Furthermore, *miR-221* levels can be obtained from blood, prostatic tissue and prostatic secretion, thus providing easily available samples (19).

Disagreement characterises the early clinical evidence, with some finding no association amongst *miR-221* downregulation and either BCR or CR (10,20), whilst others demonstrating significant correlations (8,9,16,17,45). Nonetheless, works yielding negative results suffer from methodological limitations. In one study mean follow-up was shorter than 2 years, yielding to low BCR rates (20), whereas in the other, a significant proportion of men had GS ≤6 and no PSA or clinical and pathological TNM information being available.

In our study, *miR-221* downregulation was significantly

associated with BCR on univariate analysis only but not when accounting for multiple risk factors on multivariate analysis. Also, *miR-221* was not able to predict CR and PcD.

Although it has been shown that different procedural methods such as macro-dissection or miRNA extraction may influence results (20,46), this occurrence is unlikely as we adopted the same study protocol and miRNA extraction of others showing *miR-221* significantly and well predicts adverse PCa outcomes (9,16). Conversely, other reasons may contribute to the overall absence of significant results in our cohort.

Despite approaching six years, our follow-up was relatively short and the number of patients was low overall and compared to others (9,16). Low CR and PCa death rates may have also contributed to obtain non-significant results. Overall, well established risk factors such as GS, pathological stage, number of positive nodes, positive surgical margins and PSA did not have any correlation with biochemical or CR on multivariate analysis, arguing for the need of a longer follow-up and for a higher number of patients. Other possible limitations that may have hampered the results comprise the absence of hereditary PCa records and the inclusion of men undergoing neo-adjuvant, adjuvant and salvage treatments, which may modify RP histological features (47). Furthermore, considering all three investigated molecules appear to be involved in the androgenic pathway (9,22,34), it is not known which effect hormone treatments may have had on their expression. As these are frequently used in high-risk cohorts, the effect of salvage therapies on future biomarker levels also needs to be elucidated in the future.

Finally, the assessment of a high-risk cohort is a point of strength of this study. In this group, RP alone has not proved superior to observation in reducing mortality (48). This still represents an unsolved issue as we are not able to discriminate patients who will possibly progress and die because of PCa, even after RP has been performed. Rather than a diagnostic marker that risks to enhance overtreatment, we need a prognostic marker able to predict the outcomes.

Currently, we are performing analysis using a large high-risk cohort with a >10 years follow-up to evaluate *Ki-67*, *TOPIIA* and *miR-221* ability in predicting PcD and metastasis. New analysis of the current cohort with has also been planned as a longer follow-up is indeed needed to draw stronger conclusions (9). Also, efforts must be made in increasing our genomic knowledge of PCa, as it is for breast and other cancers. In this sense "real-world" clinical-genomic database and platforms are being created and are already available to enhance high risk and advanced PCa characterisation, paving the way towards precision oncology and personalised care (49,50).

Conclusions

TOPIIA and *Ki-67* did not yield any ability in predicting BCR, CR or PcD in high-risk PCa patients who underwent RP. *miR-221* was able to predict BCR on univariate analysis only, and did not show any prognostic ability in regard to CR and PcD. Negative results may derive from a relatively short follow-up and a low number patients and events in our cohort. Further large, well-designed studies with appropriate follow-up are ongoing and needed to evaluate the ability of the investigated biomarkers in predicting risk of BCR, CR and PcD after RP in high-risk PCa.

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Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at https://tau.amegroups.com/article/view/10.21037/tau-21-628/rc

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the institutional ethics board of San Giovanni Battista Hospital

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