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ERG alterations and mTOR pathway activation in primary prostate carcinomas developing castration-resistance

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ABSTRACT

Introduction: One of the most common sites of distant metastasization of prostate cancer is bone, but to date reliable biomarkers able to predict the risk and timing of bone metastasization are still lacking.
Patients and methods: Surgically resected paraffin embedded samples from 12 primary prostate cancers that developed metachronous bone metastasis at different time points were studied (six cases within 2 years, six cases after 5 years from surgery). A targeted next-generation DNA and RNA sequencing able to assess simultaneously mutations, copy number alterations and fusion events of multiple genes was used. Immunohistochemistry was used to assess mTOR pathway activation.
Results: Rearrangements of *ETS* family genes, molecular alterations in *PTEN* and *TP53* genes were detected in 10, 6 and 5 cancers, respectively. Nine samples showed *TMPRSS2-ERG* fusions, which were associated with increased ERG expression at immunohistochemistry. mTOR pathway activation was documented in 6 patients, with a clear trend of prevalence in late-metastatic patients ($p = 0.08$).
Conclusions: A simultaneous next-generation targeted DNA and RNA sequencing is applicable on routine formalin-fixed paraffin-embedded tissues to assess the multigene molecular asset of individual prostate cancers. This approach, coupled with immunohistochemistry for ERG and mTOR pathway proteins, may help to better characterize prostate cancer molecular features with a potential impact on clinical decisions.

1. Introduction

Prostate cancer (PCa) is the third cause of cancer-related death in Europe accounting for about 10% of total cancer deaths in men [23]. The 5-year disease-specific survival rate is higher than 95% in patients with a localized or regional disease, while it drastically decreases to 30% in men with distant metastasis at the time of diagnosis [1].

Androgen-deprivation therapy (ADT) has been considered the gold standard treatment for metastatic hormonal sensitive prostate cancer (mHSPC) since the 1940s, given the androgen-dependent nature of this tumor [34]. However, the metastatic neoplasms usually progresses nine to 30 months after initial response to ADT, resulting in a status known as castration-resistant prostate cancer (CRPC) [20]. In recent years, the therapeutic arena in PCa has expanded with the introduction of new agents, including new-generation hormonal molecules (abiraterone acetate and enzalutamide), chemotherapy (cabazitaxel), and particles emitting radionuclides (Radium-223) [4,14,15,26,32,33]. They have significantly modified the natural history of mCRPC, prolonging

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survival to more than 30 months and improving patients quality of life. Moreover, the early administration of docetaxel and abiraterone acetate in mHSPC with high-volume and high-risk disease, respectively, has shown a significant survival advantage and a delay in the evolution to castration-resistant disease [16,36]. Despite these advances, mCRPC remains a disease with a lethal outcome that still needs novel therapeutic approaches to provide durable disease control and improve patient outcome.

Recent exome/whole genome sequencing analyses of prostate cancer have found genes affected by recurrent somatic mutations (*TP53*, *AR*, *APC*, *BRCA2*, *FOXA1, SPOP*), copy number alterations (*CHD1*, *PTEN*, *RB1*, *TP53, AR*) and DNA rearrangements producing fusion genes involving the ETS transcription factor family [2,5,6,17–19,30,37–39]. These studies identified the AR signaling pathway, PI3K, WNT, DNA damage repair, and cell cycle as the most altered cellular pathways in advanced prostate cancer [2,5,6,17–19,30,37–39]. Of interest, compared to localized prostate cancer, mCRPC shows a higher rate of alterations affecting TP53, and PI3K/Akt/mTOR pathways [8,30] and is characterized by clonal molecular heterogeneity [12,19].

One of the most common sites of distant metastasization of PCa is bone: skeletal metastases represent not only a therapeutic challenge, but can also dramatically affect the quality of life of mPCa [25]. To date, reliable biomarkers able to predict the risk and timing of bone metastasization in PCa are still lacking [22]. To assess whether a molecular difference exists between primary prostatic cancers that develop bone metastasis at different times of their natural history, we assessed the molecular anomalies of two groups of resected prostate cancers that developed metachronous bone metastasis at different time points during follow up. The first group of six patients developed metastasis within 2 years from surgery, and the second group of six patients developed metastasis after 5 or more years. We took advantage of recently introduced next.-generation sequencing methodologies able to assess simultaneously mutations, copy number alterations and fusion events of multiple genes using DNA from fomalin-fixed, paraffin-embedded tissues.

2. Materials and methods

2.1. Patients and samples

Twelve surgically treated primary prostate acinar adenocarcinomas that developed metachronous bone metastasis were retrieved from the ARC-Net Bio-bank at Verona University Hospital (Table 1). Six patients developed bone metastasis after more than 5 years from surgery (n.1127, 1159, 1370, 1371, 1398, 2931), the remaining six developed bone metastasis within two years from surgery (n. 1582, 1614, 1875, 1147, 1400, 1567). Eight presented a Gleason score ≥8 and a grading group ≥4, four patients had a Gleason score of 7 and a grading group ranging from 2 to 3. Informed consent was obtained from all subjects included in the study under ethics approval from the Integrated University Hospital Trust (AOUI) Program 1885 with protocol 52438 on 23 November 2010 for the collection and use of samples in the ARC-Net biobank.

2.2. Nucleic acids extraction and cDNA synthesis

DNA and total RNA were obtained by QIAamp DNA/RNA Mini Kit (Qiagen) from 15 consecutive 14-μm sections. Neoplastic cellularity was evaluated on hematoxylin & eosin (H&E) 5-μm sections every 5 sections. Purified DNA was qualified as reported previously [35] while RNA was quantified using the Qubit RNA assay kits (ThermoFisher). A minimum of 10 ng of total RNA was reverse transcribed using the SuperScript VILO cDNA Synthesis Kit according to the manufacture's instructions.

2.3. Next-generation target sequencing

Deep sequencing was performed using the Oncomine Comprehensive Panel (Thermo Fisher) in order to simultaneously analyze mutations, gene copy variations and fusion genes. In particular, the DNA panel explores selected regions, full coding DNA sequence (CDS) and copy number variation of 73, 26 and 24 cancer associated genes, respectively, while the RNA panel investigates 22 cancer fusion drivers (183 assays) (Supplementary Table 1).

The quality of the obtained libraries was evaluated by on-chip electrophoresis using the DS DNA High Sensitivity Assay kit for the Agilent 2100 Bioanalyzer System (Agilent Technologies). Emulsion PCR and chip loading were performed using Ion Chef system (ThermoFisher). Sequencing was carried out on a 318 chip in the Ion Torrent Personal Genome Machine (ThermoFisher).

After sequencing, unaligned BAM files were transferred to the Ion Reporter Software 5.0 and analyzed using the Oncomine Variants (5.0) filter/workflow. Data analysis, including alignment to the hg19 human reference genome and variant calling, was performed using the Torrent Suite Software ver. 4.0 (Life Technologies). Filtered variants were annotated using a custom pipeline based on vcflib (https://github.com/ekg/vcflib), SnpSift [10], the Variant Effect Predictor (VEP) software [24] and NCBI RefSeq database. Alignments were visually verified with the Integrative Genomics Viewer (IGV) v2.3 [31].

2.4. Immunohistochemistry (IHC)

Briefly, 4 μm formalin-fixed and paraffin-embedded (FFPE) serial sections were incubated overnight at 4 °C with primary antibodies: anti-ERG (1:200 dilution, Biocare Medical), anti-ph-mTOR (1:1000 dilution;

Table 1
Pathological and molecular findings in PCa.

Sample	Age	Gleason Score	Grading group	Time of bone metastasis	*PTEN*[a]	*TP53*[a]	NGS fusion	qRT-PCR fusion	ERG	ph-mTOR	ph-p70S6K	ph-4EBP1
1127	64	7	3	> 5 yrs	wt	wt	TMPRSS2-ERG	Yes	3+	0	0	0
1159	57	8	4	> 5 yrs	LOH	BI	TMPRSS2-ERG[b]	Yes	3+	2+	2+	1+
1370	67	9	5	> 5 yrs	HD	HD	TMPRSS2-ERG[b]	Yes	3+	2+	3+	2+
1371	61	9	5	> 5 yrs	wt	wt	No	No	3+	3+	3+	2+
1398	54	7	2	> 5 yrs	wt	wt	TMPRSS2-ERG[b]	Yes	1+	2+	2+	2+
2931	65	9	5	> 5 yrs	BI	LOH	TMPRSS2-ERG[b]	Yes	3+	3+	2+	3+
1582	75	9	5	< 2 yrs	wt	wt	No	No	0	0	0	0
1614	63	8	4	< 2 yrs	wt	wt	TMPRSS2-ERG[b]	Yes	3+	0	0	0
1875	61	8	4	< 2 yrs	wt	LOH	No	No	0	0	0	0
1147	75	9	5	< 2 yrs	HD	wt	TMPRSS2-ERG[b]	Yes	2+	0	0	0
1400	61	7	2	< 2 yrs	LOH	wt	TMPRSS2-ERG[b]	Yes	0	0	0	0
1567	69	7	2	< 2 yrs	LOH	LOH	TMPRSS2-ERG[b]	Yes	3+	2+	2+	1+

[a] wt, wild type; LOH, Loss of heterozygosity; BI, Biallelic inactivation trough LOH and somatic mutation; HD: homozygous deletion.
[b] Multiple fusions.

EPITOMICS), anti-ph-4EBP1 (Thr37/46, clone 236B4; Cell Signaling) and anti-ph-p70S6K (Thr389, clone 1A5; Cell Signaling). Primary antibodies were then detected by 30 min incubation with HRP-labeled secondary antibody (Leica Microsystem, PV6119) followed by detection with 3,3' diaminobenzidine (Dako). The immunolabeled slides were reviewed and scored by two pathologists (M.F. and M.B.), blinded to histopathological, molecular and clinical variables. Appropriate positive and negative controls were run concurrently. A H-score system was established for ERG, ph-mTOR and its phosphorylated downstream effectors 4EBP1 and p70S6K as reported previously [9]. In particular, the H-score was calculated by multiplying the intensity of the stain (0: no staining; 1: weak staining; 2: moderate staining; 3: intense staining) by the percentage of positive tumor cells (ranging from 0 to 100) [9]. A four-tier score based on the Hscore was then defined for ERG, ph-mTOR and ph-4EBP1 staining: 0 (0 < H-score < 49), 1+ (50 < H-score > 99), 2+ (100 < H-score > 199) and 3+ (200 < H-score > 300). A similar four-tier score, based on the percentage of positive tumor cells, was also calculated for ph-p70S6K expression: 0 (< 10%), 1+ (10%–33%), 2+ (34%–66%) and 3+ (> 66%).

2.5. Quantitative RT-PCR for ETS fusions

Total RNA was extracted using the All Prep DNA/RNA Mini Kit (Qiagen). RNA quality was verified by RNA 6000 Nano kit on Agilent 2100 Bioanalyzer (Agilent Technologies), while RNA concentration was determined by Nanodrop at 260 nm. 1 µg of DNase-treated RNA was reverse transcribed using SuperScript® VILO cDNA Synthesis Kit (Life Technologies) and High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) in a volume of 20 µl according to the manufacture's instructions. TaqMan analysis was performed in triplicate using the predesigned assays for TMPRSS2-ERG.T2E4.COSF28 (Hs0439636), TMPRSS2-ERG.T1E4.COSF38 (Hs03063375), TMPRSS2-ERG.T2E5.COSF29 (Hs04396367), TMPRSS2-ERG.T1E5.COSF26 (Hs04396339), and TMPRSS2-ERG.T2E2.COSF27 (Hs04396356).

3. Results

3.1. Prevalence of driver fusions, gene mutations and copy number alterations in mPCa

Nine of 12 (83%) samples harbored driver fusions involving *ETS* family genes consisting of *TMPRSS2-ERG* fusions (Fig. 1). Multiple *TMPRSS2-ERG* fusion variants were found in individual samples, with 8 cases showing two or more concurrent variants. In particular, a fusion involving exon 1 of *TMPRSS2* and exon 4 of *ERG* (TMPRSS2(1)-ERG(4)) was found in all 9 samples (100%), followed by TMPRSS2(1)-ERG(2), TMPRSS2(1)-ERG(5) and TMPRSS2(2)-ERG(4) that were each detected in 6 of 9 (67%) samples (Fig. 1).

A total of 12 somatic mutations (10 missense and 2 nonsense) involving *TP53, PTEN, SMAD4, APC, CDH1, PIK3CA, SPOP, FGFR4, BCL9, MED12* and *NF1* were detected (Fig. 1 and Supplementary Table 2). In 7 of 12 cases (58.3%), at least one somatic mutation was detected, and 3 of 7 cases (42.8%) displayed multiple driver gene somatic mutations. *APC* was the only gene mutated in multiple cases.

PTEN and *TP53* were the genes most frequently affected by copy number alteration in our series. In particular, *PTEN* showed loss of heterozygosity (LOH) or homozygous deletion (HD) in 4 of 12 (33%) and 2 of 12 (17%) of cases, respectively. *TP53* displayed LOH in 4 of 12 (33%) and HD in 1 of 12 (8%). Biallelic inactivation of *PTEN* and *TP53* due to somatic mutation and LOH was found in 1 patient each. *SMAD4* was also altered in 3 of 12 (25%) cases.

No differences in *TP53* and *PTEN* alterations were found between the groups of early and late-metastatic patients.

3.2. TMPRSS2-ERG fusion variants validation is consistent with ERG expression

Five *TMPRSS2-ERG* fusion variants identified by next generation sequencing were selected for validation by qRT-PCR (Fig. 2). Selection of the fusion variants was based on the availability of commercial assays for the detection. The experiments were performed independently using two different cDNA synthesis kits (SuperScript® VILO cDNA Synthesis Kit and High-Capacity cDNA Reverse Transcription Kit) to avoid the possibility of false positive/negative due to the cDNA synthesis method used. Quantitative RT-PCR confirmed deep sequencing results for the five *TMPRSS2-ERG* fusion variants.

ERG immunohistochemical overexpression was observed in 8 of 9 TMPRSS2-ERG cases (Fig. 2c and Table 1) while one case that showed no *TMPRSS2-ERG* fusion by NGS was strongly expressed by IHC.

No differences in ERG fusions prevalence were found between the groups of early and late-metastatic patients.

3.3. mTOR pathway activation in early and late mPCa patients

Activation of the mTOR pathway was assessed by evaluating the phosphorylated forms of mTOR and its downstream effectors 4EBP1 and p70S6K (Fig. 3). An increased expression of activated mTOR and of its downstream effectors was found in 6 (50%) cases, including 5 of 6 late metastatic patients and 1 of six 6 early metastatic patients. Although this difference does not reach statistical significance, an evident trend of prevalence of the activation of mTOR pathway was observed in the group of late metastatic patients (Fisher's exact test, $p = 0.08$). No relationship was observed between mTOR pathway activation and other molecular alterations.

4. Discussion

Prostate cancer is characterized by a wide spectrum of clinical behavior, varying from indolent forms up to aggressive metastatic lethal tumors, denoting the potentially extreme genomic heterogeneity of this disease. This may significantly affect the personalization of treatment based on the genomic asset of the individual cancers.

In this study, we interrogated 12 surgically treated primary prostate cancers that developed bone metastasis for the presence of mutations and copy number variations in known cancer-related genes, as well as for the mRNA expression of known fusion genes, using a targeted next-generation sequencing approach permitting to analyze simultaneously the mutational status, copy number alterations and fusion events of multiple genes. We observed: (i) a mutational asset comparable with that described in current literature on PCa; (ii) a high frequency of *TMPRSS2-ERG* fusion variants, which corresponded to ERG protein immunohistochemical overexpression; (iii) a frequency of activation of mTOR pathway in late bone metastasizing cancers higher than in cancers developing early bone metastasis.

Seven of 12 (58.3%) tumors harbored at least one driver gene mutation and 25% (3/12) showed concurrent mutations in two or more genes. *PTEN* was the most frequently altered gene (50%) followed by *TP53* (42%). These results are concordant with those reported in prior studies, which associated these molecular alterations with prostate cancer aggressiveness [5,17,29].

Fusion genes involving *ETS* family were found in 10 of 12 cases (83%). It is well established that *ETS* genes play a central role in prostate cancer, most commonly through the fusion of the androgen-regulated promoter element *TMPRSS2* leading to induction of cell proliferation and increase of cell invasion [38,40].

Multiple *TMPRSS2-ERG* fusion variants were found in 8 individual samples, which is consistent with existing data [11,30]. The identification of multiple fusions in individual samples may be due to either alternative splicing occurring after a single genomic event or independent events occurring in different tumor clones [12,21,30].

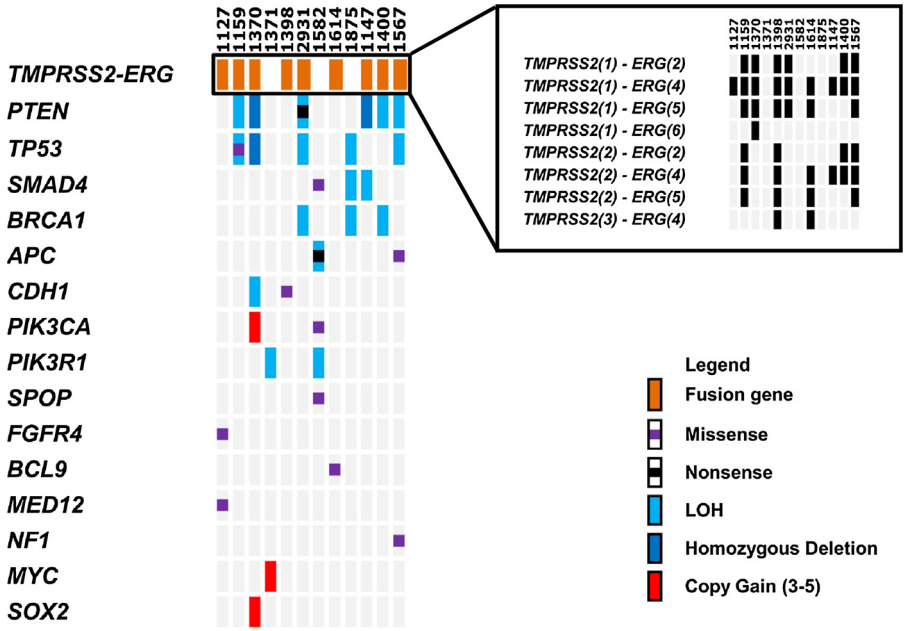

Fig. 1. Recurrent genetic alterations observed in 12 PCa. Genes are listed based on frequency of alterations from top to bottom. Box displays *TMPRSS2-ERG* fusion variants detected in each sample.

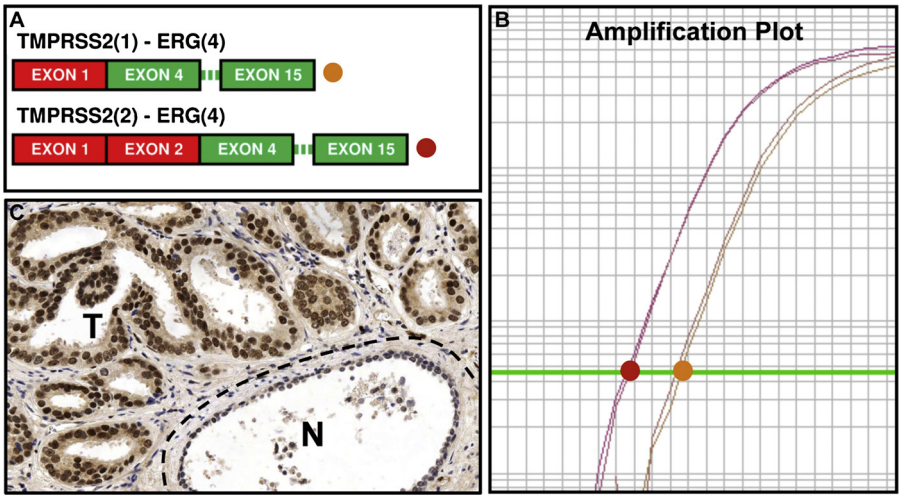

Fig. 2. Validation of *TMPRSS2-ERG* fusion by qRT-PCR. Sample number 1147 showed two fusions variants by NGS (A) that were validated by qRT-PCR (B) and were associated to increased ERG expression by IHC (C) in neoplastic tissue (T) compared to normal adjacent tissue (N).

Of note, our NGS data for *TMPRSS-ERG* fusion variants matched well with qRT-PCR and IHC results, supporting the efficiency of the next generation targeted sequencing method and the accuracy of ERG immunohistochemical expression as surrogate for defining *TMPRSS-ERG* status [9]. Nevertheless, two cases (1371 and 1400) were differently classified by IHC as positive and negative respectively. The high expression of *ERG* without detectable *TMPRSS2-ERG* fusions by NGS could be attributed to the presence of an *ERG* fusion with other genes such as *NDRG1*, or *FKBPS* [9,27,28] that are not included in our targeted panel, while the negative case could be related to *ERG* fusion heterogeneity within the same prostate tumor [3,9].

The activation of PI3K/Akt/mTOR signaling was assessed by IHC, as the involvement of this pathway in the progression of prostate cancer to CRPC is well documented [7,30,37,39]. We found the activation of mTOR and its downstream effectors 4EBP1 and p70S6K in 6 of 12 (50%) of our patients. Namely, five of six late metastasizing and one of six early metastasizing cases. The presence of a trend of prevalence of the activation of the mTOR pathway in late bone metastatic PCa patients warrants further studies, since new molecular biomarkers able to predict the risk and the timing of bone metastasization are urgently needed.

Notably, in a portion of cases alterations of *PTEN* and *PIK3CA* genes represented the molecular basis of the aberrant activation of PI3K/Akt/mTOR pathway. Several PI3K/AKT/mTOR signaling inhibitors are currently under investigation in CRPC, either alone or in combination with other molecules, our data suggest that immunostaining may be used as a surrogate for detection of mTOR pathway activation. In particular, the dual inhibition of PI3K/Akt/mTOR and AR signaling represents a promising anticancer strategy in the subset of prostate tumors with *PTEN* inactivation, given the linear relationship between the PI3K/AKT/mTOR and androgen receptor (AR) pathways (hyper-activation of PI3K/Akt/mTOR pathway suppresses AR transcriptional output and stability, and AR blockade activates Akt) [13]. Of note, *PTEN* loss has been addressed as a potential predictor of response to ipatasetib (an oral Akt inhibitor) plus abiraterone in mCRPC patients [13]. The value of *PTEN* loss as a predictive biomarker of response to

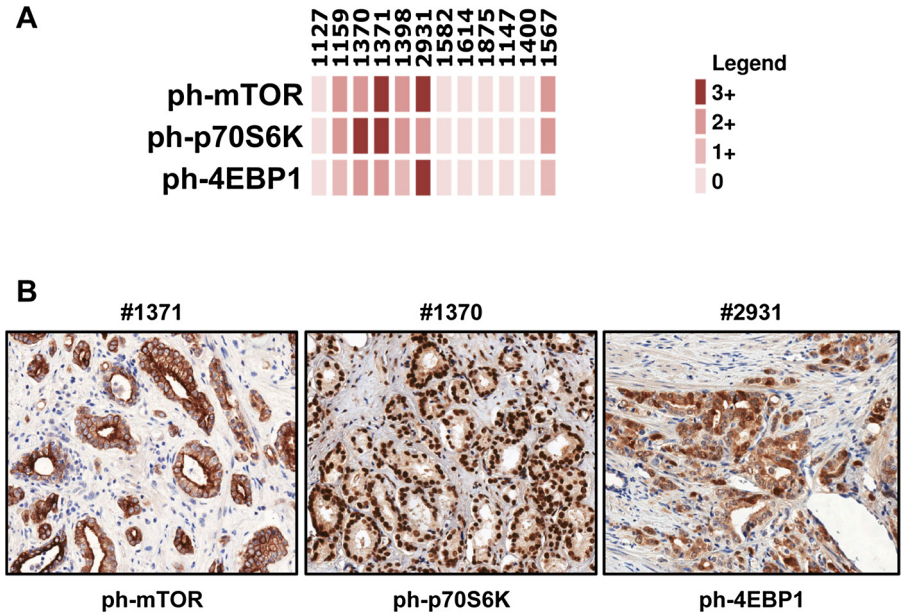

Fig. 3. Immunohistochemical profile of mTOR pathway in PCa. (A) Summary of mTOR pathway activation in our PCa series. (B) Representative examples of immunohistochemical staining for mTOR pathway components in PCa samples. Original magnfications, 20×.

dual PI3K/Akt/mTOR and AR pathways inhibition represents an important step towards personalized therapy, which deserves dedicated investigation.

5. Conclusions

Our study shows that a simultaneous next-generation targeted DNA and RNA sequencing on routine formalin-fixed paraffin-embedded tissues, coupled with immunohistochemistry, may help to better characterize PCa molecular features with a potential impact on clinical decisions. The analysis of mTOR pathway appears as a promising target in our cohort, although the small sample size in our exploratory study cannot support any definitive conclusions.

Potential competing interests

The authors have no competing interests to declare.

Authors' contributions

R.T.L., C.V., A.S., conceived the study. M.S. V.C., designed the validation experiment. R.T.L. coordinated patients and samples data management and supervised ethical protocols. R.I, A.B.P, F.B collected materials and clinical data. C.L., B.R, M.B. analysed histopathological data. C.C, D.A. carried out deep sequencing and raw data analysis. A.M., M.S. performed bioinformatic analysis. M.F, C.V. analysed immunohistochemistry. C.V, V.C, C.L., M.F, A.S. drafted the manuscript. R.T.L. finalized the manuscript. All authors approved the final version of the manuscript.

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