Pathology - Research and Practice xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

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

Caterina Vicentini[a,b,1], Cinzia Cantù[a,1], Davide Antonello[a,c], Michele Simbolo[a,b], Andrea Mafficini[a], Claudio Luchini[b], Borislav Rusev[a,b], Antonio Benito Porcaro[e], Roberto Lacovelli[f], Matteo Fassan[g], Vincenzo Corbo[a,b], Matteo Brunelli[b], Walter Artibani[d], Aldo Scarpa[a,b,*], Rita T. Lawlor[a]

[a] *ARC-NET Research Centre, University of Verona, Verona, Italy*
[b] *Department of Diagnostics and Public Health, Section of Anatomical Pathology, University and Hospital Trust of Verona, Verona, Italy*
[c] *Department of Surgery, General Surgery B, University of Verona, Verona, Italy*
[d] *Urologic Unit, AOUI, Verona, Italy*
[e] *Department of Internal Medicine, APOUI, Verona, Italy*
[f] *Oncology Unit, AOUI, Verona, Italy*
[g] *Department of Medicine (DIMED), University of Padua, Padua, PD, Italy*

ARTICLE INFO

*Keywords:*
Prostate cancer
Bone metastasis
Castration resistance
Next-generation sequencing
Multigene panels

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

* Corresponding author at: ARC-Net Research Center, Department of Diagnostics and Public Health – Section of Pathology, University and Hospital Trust of Verona, Piazzale L.A Scuro, 10, Piastra Odontoiatrica (II floor), 37134 Verona, VR, Italy.
*E-mail address:* aldo.scarpa@univr.it (A. Scarpa).
[1] Shared first authorship.

https://doi.org/10.1016/j.prp.2018.08.031
Received 28 June 2018; Received in revised form 20 August 2018; Accepted 28 August 2018

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.