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Prostate Cancer

# Multifocal Primary Prostate Cancer Exhibits High Degree of Genomic Heterogeneity

*Marthe Løvf*[a], *Sen Zhao*[a], *Ulrika Axcrona*[b], *Bjarne Johannessen*[a,c], *Anne Cathrine Bakken*[a], *Kristina Totland Carm*[a], *Andreas M. Hoff*[a], *Ola Myklebost*[c,d,e], *Leonardo A. Meza-Zepeda*[d,f], *A. Kathrine Lie*[b], *Karol Axcrona*[g], *Ragnhild A. Lothe*[a,c], *Rolf I. Skotheim*[a,c,*]

[a] *Department of Molecular Oncology, Institute for Cancer Research, Oslo University Hospital-Radiumhospitalet, Oslo, Norway;* [b] *Department of Pathology, Oslo University Hospital-Radiumhospitalet, Oslo, Norway;* [c] *Norwegian Cancer Genomics Consortium, Oslo, Norway;* [d] *Department of Tumor Biology, Institute for Cancer Research, Oslo University Hospital-Radiumhospitalet, Oslo, Norway;* [e] *Department of Clinical Science, University of Bergen, Bergen, Norway;* [f] *Genomics Core Facility, Department of Core Facilities, Institute for Cancer Research, Oslo University Hospital-Radiumhospitalet, Oslo, Norway;* [g] *Department of Urology, Akershus University Hospital, Lørenskog, Norway*

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**Abstract**

***Background:*** Most primary prostate cancers are multifocal with individual tumors harboring different aggressiveness; however, the genomic heterogeneity among these tumors is poorly understood.
***Objective:*** To better understand the biological basis for clinical variability among different lesions, we sought to comprehensively characterize the heterogeneity of somatic gene mutations in multifocal prostate cancer.
***Design, setting, and participants:*** High-coverage whole-exome sequencing of 153 frozen tissue samples, taken from two to three distinct tumor foci and one non-cancerous area from each of 41 patients, covering a total of 89 tumor foci.
***Outcome measurements and statistical analysis:*** State-of-the-art bioinformatics tools for mutation calling and copy number determination from whole-exome sequencing data.
***Results and limitations:*** We found a very high degree of interfocal heterogeneity among tumors, that is, 76% of pairwise-compared tumor foci from the same prostatectomy specimen had no point mutations in common and DNA copy number changes were rarely shared across cancer foci. The few point mutations shared across tumor foci were seldom in cancer-critical genes.
***Conclusions:*** In this first large genomic heterogeneity study of primary prostate cancer, we observe that different tumor foci within the same patient are genetically distinct, only rarely sharing any somatic gene mutations, including those in cancer driver genes. This heterogeneity affects how genomics-based management of prostate cancer can be implemented, as information from all tumor foci is necessary to draw valid conclusions about the cancer's genomic alterations.
***Patient summary:*** Most primary prostate cancers consist of multiple tumors within the same organ, but little is known about their relationships. We have compared the sets of gene mutations among such tumors and found that they only exceptionally have any in common. This will influence treatment decisions in the future as each tumor's mutations will render it unique and have to be considered to gain the best treatment results.


* Corresponding author. Department of Molecular Oncology, Institute for Cancer Research, Oslo University Hospital-Radiumhospitalet, P. O. Box 4953 Nydalen, 0424 Oslo, Norway.
Tel. +47 22781727; Fax: +47 22781745.
E-mail address: rolf.i.skotheim@rr-research.no (R.I. Skotheim).

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## 1. Introduction

Prostate cancer is the most common cancer type among men in the Western world [1]. Although some of these cancers are slow-growing, others are more aggressive and prostate cancer is annually responsible for a quarter million deaths world-wide [2]. Therefore, a pressing demand in the management of this disease is the identification of patients in need of aggressive follow-up. This decision is complicated by the multifocal nature of the disease, with the vast majority of patients having more than one tumor focus [3]. These individual tumor foci may have different aggressiveness and progress independently of one another [4,5]. Although recently published projects have sequenced large series of prostate cancers, with the aim to identify the most commonly mutated genes, these projects have drawn a relatively simplified and uniform picture of the disease by only including one malignant sample per patient [6–11]. Since the majority of prostate cancers are multifocal and different driver mutations can be active within different tumor foci [12], there is a great need for more thorough investigation of prostate cancer heterogeneity. A few studies addressing the multifocality with high-throughput sequencing have been published, but these are limited by analyses of very few patients (2–5 patients) [13–16], and controversy remains with regards to how prostate cells evolve and produce individual cancer foci. Therefore, and for the first time, a large cohort of multifocal prostate cancers was submitted to high-coverage whole-exome sequencing. On analyses of these data, we observed an exceptionally large degree of interfocal heterogeneity, which will have implications both for clinical decision-making and design of prostate cancer studies in the future.

## 2. Materials and methods

### 2.1. Patient material

Cancer and benign samples used in this study were selected from a consecutive cohort of 571 patients who underwent radical prostatectomy at Oslo University Hospital-Radiumhospitalet during 2010–2012. The patient cohort mainly comprised of intermediate- and high-risk cancer patients. From each of the prostatectomy specimens, three to seven tissue samples (average of 5.5) were taken from different sections and areas of the prostate and freshly frozen. The location of each sample within the prostate was registered, and the area around were assessed by an uropathologist to set the Gleason score and register within which tumor focus the sample was taken. For the current study, 153 samples from 89 tumors within 41 different patients were carefully selected to enrich for samples deriving from multiple foci per patient (Table 1). Only samples from clearly separated foci (>2–4 mm apart) were chosen for sequencing.

DNA and RNA were isolated using the AllPrep DNA/RNA/miRNA Universal kit (Qiagen, Germany), according to the manufacturer's protocol.

### 2.2. Whole-exome sequencing

A total of 153 samples (112 tumors and 41 benign tissues) were analyzed with exome sequencing. Sequencing reads were aligned to the human reference genome and single nucleotide variants and DNA copy number alterations identified. For a detailed description of the analysis pipeline for sequencing reads, see Supplementary methods. The exome sequencing data are being deposited to the European Genome-phenome Archive (www.ega-archive.org).

### 2.3. TMPRSS2-ERG fusion transcript analysis

Expression of the fusion gene *TMPRSS2-ERG* was investigated using reverse transcriptase polymerase chain reaction (RT-PCR), with primers flanking the common breakpoints (Supplementary Table 1; Supplementary methods).

## 3. Results

### 3.1. The landscape of multifocal prostate cancer

To investigate the heterogeneity of somatic gene mutations in multifocal prostate cancer, we performed high-coverage exome sequencing of 153 samples, taken from two to three distinct tumor foci and one non-cancerous prostate tissue sample from each of 41 patients, covering a total of 89 tumor foci (Table 1). Each tumor focus was represented by one to two samples to facilitate analysis of both inter- and intrafocal heterogeneity. A median coverage depth of 239× (Q1 = 218, Q3 = 256) per tumor sample and 93× (Q1 = 86, Q3 = 106) for non-cancerous samples were achieved, with at least 80% of the targeted bases having a coverage of 100× or more in all tumor samples (Supplementary Table 2). This is higher coverage than in previously published exome sequencing studies on prostate cancer. To evaluate the representativeness of our data, we compared general mutational patterns with those in previously published studies [6,9] before investigating mutational heterogeneity. We identified altogether 4084 somatic mutations (Supplementary Table 3) and observed the same type of mutational signature as previously published for primary prostate cancer (Supplementary Fig. 1) [9]. Of the 4084 mutations, 1378 occurred in one highly mutated tumor sample. This sample harbored a non-synonymous mutation (T881A) in the exonuclease *POLE*, which is likely to explain the high mutation rate. The highly mutated sample was excluded from the significance analysis of somatically mutated genes, but included in the remaining analyses, tables, and figures. The other 111 tumor samples harbored 24 mutations (range, 0–98) on average, which corresponds to 0.48 mutations per megabase targeted DNA sequence. The remaining 2706 mutations involved 2344 genomic positions within 1971 unique genes (Supplementary Table 4).

We searched for mutations in genes likely to contribute to carcinogenesis by utilizing two different algorithms, selected based on their ability to identify mutations at a higher frequency than expected (MutSig [17]) and with a potential functional consequence (Oncodrive-fm [18]; Supplementary Table 5). We considered the top 20 genes from both algorithms and focused on those recurrently mutated in multiple patients, adding up to 27 unique genes (Fig. 1; Supplementary Fig. 2). Selected mutations were

**Table 1 – Patients and samples in prostate cancer cohort. Left, information about each patient with Gleason score (GS) for the prostate, pathologic tumor stage (pT), pathologic lymph node stage, if free margins were observed after prostatectomy, the presence of biochemical recurrence. Middle, information about each tumor focus (GS and pT) and indication of which tumor focus is the index tumor. Right, information about each sample, with GS, whether benign tissue was present in the surrounding area, the corresponding focus number and which sample is located in areas with extraprostatic extension**

| | Patient | | | | | Focus 1 | | Focus 2 | | Focus 3 | | | Sample 1 | | | Sample 2 | | | Sample 3 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ID | GS | pT | pN | Free margin | BCR | GS | pT | GS | pT | GS | pT | Index | GS | BTP | Focus | GS | BTP | Focus | GS | BTP | Focus | EPE |
| 1 | 3 + 4 | T3a | N0 | Yes | No | 3 + 3 | 2 | 3 + 4 | 3a | – | – | F2 | 3 + 4 | No | F1 | 3 + 4 | No | F1 | 3 + 4 | No | F2 | S3 |
| 2 | 3 + 4 | T3a | NX | Yes | No | 3 + 4 | 3a | 3 + 3 | 2 | – | – | F1 | 3 + 4 | No | F1 | 3 + 4 | No | F1 | 3 + 4 | No | F2 | S2 |
| 3 | 3 + 4 | T3a | NX | Yes | No | 3 + 4 | 3a | 3 + 3 | 2 | – | – | F1 | 3 + 4 | No | F1 | 3 + 3 | No | F1 | 3 + 3 | Yes | F2 | S1 |
| 4 | 4 + 4 | T3a | N0 | No | No | 4 + 4 | 3a | 4 + 3 | 3a | – | – | F1 | 3 + 4 | Yes | F1 | 4 + 4 | No | F1 | 3 + 4 | Yes | F2 | S1 |
| 5 | 4 + 3 | T3a | N0 | Yes | No | 4 + 3 | 3a | 4 + 3 | 3a | – | – | F1 | 4 + 4 | Yes | F1 | 4 + 4 | No | F1 | 3 + 3 | Yes | F2 | S1,S3 |
| 6 | 3 + 4 | T3b | N0 | No | No | 3 + 4 + 5 | 3b | 3 + 4 | 3a | – | – | F1 | 3 + 4 | No | F2 | 3 + 4 | Yes | F1 | 3 + 3 | No | F2 | S1,S3 |
| 7 | 4 + 3 | T3a | NX | Yes | No | 4 + 3 | 3a | 3 + 3 | 2 | – | – | F1 | 3 + 4 | No | F1 | 4 + 3 | No | F1 | 3 + 3 | Yes | F2 | S1,S2 |
| 8 | 4 + 4 | T3a | NX | Yes | No | 4 + 4 | 3a | 3 + 4 | 2 | – | – | F1 | 4 + 4 | Yes | F1 | 4 + 3 | No | F1 | 3 + 4 | No | F2 | S1 |
| 9 | 3 + 4 | T3a | NX | Yes | No | 3 + 4 | 2 | 3 + 4 | 3a | – | – | F2 | 3 + 4 | No | F1 | 3 + 4 | Yes | F2 | 3 + 4 | No | F1 | S2 |
| 10 | 4 + 5 | T3a | NX | Yes | No | 4 + 5 | 3a | 4 + 5 | 2 | – | – | F1 | 4 + 4 | No | F1 | 4 + 4 | No | F1 | 4 + 4 | No | F2 | S1,S2 |
| 11 | 4 + 3 | T3a | N1 | Yes | Yes | 4 + 3 + 5 | 3a | 3 + 3 | 2 | – | – | F1 | 4 + 3 | No | F1 | 4 + 3 | No | F1 | 3 + 3 | Yes | F2 | S1 |
| 12 | 4 + 3 | T3b | NX | Yes | No | 4 + 3 | 3b | 3 + 4 | 2 | 4 + 3 | 2 | F1 | 4 + 3 | No | F1 | 3 + 4 | No | F2 | 4 + 4 | Yes | F3 | – |
| 13 | 3 + 4 | T3a | NX | Yes | No | 3 + 4 | 2 | 3 + 3 | 2 | 3 + 4 | 3a | F3 | 4 + 3 | No | F1 | 3 + 3 | No | F2 | 3 + 4 | Yes | F3 | S3 |
| 14 | 3 + 4 | T3a | NX | Yes | Yes | 3 + 4 | 3a | 3 + 4 | 2 | 3 + 3 | 2 | F1 | 3 + 4 | No | F1 | 3 + 3 | Yes | F2 | 3 + 3 | Yes | F3 | S1 |
| 15 | 4 + 3 | T3a | NX | No | Yes | 4 + 3 + 5 | 2 | 3 + 4 | 2 | – | – | F1 | 4 + 4 | No | F1 | 3 + 4 | No | F2 | 3 + 3 | No | F2 | – |
| 16 | 3 + 5 | T3a | N0 | Yes | Yes | 4 + 4 | 2 | 4 + 5 | 3a | – | – | F2 | 4 + 4 | Yes | F1 | 4 + 5 | No | F2 | 4 + 5 | No | F2 | – |
| 17 | 3 + 4 | T3b | NX | Yes | No | 3 + 3 | 2 | 3 + 4 | 2 | – | – | F2 | 3 + 4 | No | F1 | 3 + 4 | No | F1 | 3 + 3 | No | F2 | – |
| 18 | 3 + 5 | T3a | N0 | Yes | Yes | 3 + 5 | 3a | 3 + 4 | 2 | – | – | F1 | 5 + 4 | No | F1 | 3 + 4 | No | F1 | 3 + 4 | No | F2 | – |
| 19 | 3 + 4 | T3a | NX | Yes | No | 3 + 4 | 3a | 3 + 4 | 2 | – | – | F1 | 3 + 3 | Yes | F2 | 3 + 4 | No | F1 | 3 + 4 | No | F2 | – |
| 20 | 3 + 4 | T3a | N0 | Yes | No | 3 + 3 | 2 | 3 + 4 | 2 | – | – | F2 | 3 + 3 | No | F1 | 3 + 3 | No | F2 | 3 + 3 | No | F2 | – |
| 21 | 3 + 4 | T3a | N0 | Yes | No | 3 + 4 | 3a | 4 + 3 | 2 | – | – | F1 | 3 + 3 | No | F1 | 3 + 4 | No | F1 | 3 + 4 | No | F2 | – |
| 22 | 5 + 3 | T3a | NX | Yes | No | 5 + 3 | 3a | 3 + 4 | 2 | – | – | F1 | 3 + 3 | Yes | F1 | 4 + 3 | No | F2 | 3 + 3 | No | F2 | – |
| 23 | 4 + 3 | T3b | NX | No | Yes | 3 + 4 | 2 | 4 + 3 | 3a | – | – | F2 | 3 + 3 | No | F1 | 3 + 3 | No | F1 | 3 + 3 | Yes | F2 | – |
| 24 | 4 + 4 | T3a | NX | No | Yes | 4 + 4 | 3a | 3 + 4 | 2 | – | – | F1 | 4 + 4 | No | F1 | 4 + 4 | No | F1 | 3 + 3 | Yes | F2 | – |
| 25 | 3 + 4 | T3a | N0 | No | Yes | 3 + 4 | 3a | 3 + 4 | 2 | – | – | F1 | 3 + 3 | No | F1 | 3 + 3 | Yes | F2 | 3 + 3 | No | F2 | – |
| 26 | 3 + 4 | T3a | NX | No | No | 3 + 4 | 2 | 3 + 4 | 3a | – | – | F2 | 3 + 4 | No | F1 | 3 + 3 | Yes | F2 | 3 + 4 | No | F2 | – |
| 27 | 3 + 4 | T2c | NX | No | No | 3 + 4 | 2 | 3 + 3 | 2 | 3 + 3 | 2 | – | 3 + 4 | No | F1 | 3 + 3 | Yes | F2 | 3 + 4 | Yes | F3 | – |
| 28 | 4 + 5 | T2c | NX | Yes | No | 3 + 4 | 2 | 4 + 5 | 2 | 3 + 4 | 2 | F2 | 3 + 3 | No | F1 | 3 + 4 | No | F2 | 3 + 4 | No | F3 | – |
| 29 | 4 + 3 | T2c | N0 | Yes | No | 4 + 3 + 5 | 2 | 3 + 4 | 2 | 3 + 4 | 2 | F1 | 3 + 4 + 5 | No | F1 | 3 + 3 | No | F2 | 3 + 3 | Yes | F3 | – |
| 30 | 3 + 4 | T2c | NX | Yes | No | 3 + 4 | 2 | 3 + 3 | 2 | 3 + 4 | 2 | F1 | 3 + 4 | No | F3 | 3 + 4 | No | F1 | 3 + 4 | Yes | F2 | – |
| 31 | 3 + 4 | T2c | NX | Yes | No | 3 + 3 | 2 | 3 + 4 | 2 | – | – | F2 | 3 + 4 | No | F1 | 3 + 4 | No | F2 | – | – | – | – |
| 32 | 3 + 4 | T2c | N0 | Yes | No | 3 + 4 | 2 | 3 + 4 | 2 | – | – | – | 3 + 4 | No | F1 | 3 + 4 | No | F2 | – | – | – | – |
| 33 | 3 + 4 | T2c | NX | Yes | No | 3 + 4 | 2 | 3 + 4 | 2 | – | – | F1 | 3 + 4 | No | F2 | 3 + 4 | No | F1 | – | – | – | – |
| 34 | 3 + 3 | T2c | NX | Yes | No | 3 + 3 | 2 | 3 + 3 | 2 | – | – | F1 | 3 + 3 | No | F1 | 3 + 3 | No | F2 | – | – | – | – |
| 35 | 3 + 4 | T2c | NX | Yes | No | 3 + 4 + 5 | 2 | 3 + 4 | 2 | – | – | F1 | 3 + 4 | No | F1 | 3 + 4 | Yes | F2 | – | – | – | – |
| 36 | 3 + 4 | T2c | NX | Yes | No | 3 + 4 + 5 | 2 | 3 + 3 | 2 | – | – | F1 | 3 + 3 | No | F1 | 3 + 3 | No | F2 | – | – | – | – |
| 37 | 3 + 3 | T2c | NX | Yes | No | 3 + 4 | 2 | 3 + 4 | 2 | – | – | F1 | 3 + 3 | No | F1 | 3 + 3 | No | F2 | – | – | – | – |
| 38 | 3 + 3 | T2b | NX | Yes | No | 3 + 3 | 2 | 3 + 4 | 2 | – | – | F2 | 3 + 3 | No | F1 | 3 + 3 | No | F2 | – | – | – | – |
| 39 | 3 + 4 | T2c | N0 | Yes | No | 3 + 3 | 2 | 3 + 4 + 5 | 2 | – | – | F2 | 3 + 3 | Yes | F1 | 3 + 4 | No | F2 | – | – | – | – |
| 40 | 3 + 4 | T2c | NX | Yes | No | 3 + 4 | 2 | 3 + 4 | 2 | – | – | F1 | 3 + 3 | No | F1 | 3 + 3 + 4 | No | F2 | – | – | – | – |
| 41 | 3 + 4 | T2c | N0 | Yes | No | 3 + 3 | 2 | 3 + 3 | 2 | – | – | – | 3 + 3 | Yes | F1 | 3 + 3 | No | F2 | – | – | – | – |

BCR = biochemical recurrence; BTP = benign tissue present; EPE = extraprostatic extension; GS = Gleason score; pN = pathologic lymph node stage; pT = pathologic tumor stage.

validated with PCR (Supplementary Fig. 3). Among the 27 genes, we found genes that have previously been implicated in prostate cancer, such as *MED12*, *FOXA1*, *SPOP*, *SMARCA1*, and *CTNNB1* [6,7,9,10], in addition to novel genes. Given the increased interest in DNA damage repair genes, we specifically looked for somatic mutations in *BRCA1/2* and DNA mismatch repair genes. We observed no *BRCA1* mutations; however, *BRCA2* was mutated in five samples from three tumors. We also observed *MSH6* and *MSH4* mutations in one sample each. Interestingly, all samples containing mutations in these genes had more mutations than the average.

One or more mutations in the top-scoring genes were found in approximately half of the tumor samples investigated (55/112). Interestingly, RT-PCR analysis of the known fusion gene *TMPRSS2-ERG* revealed that samples without mutations in these genes more commonly expressed the fusion gene than samples with mutations (36/57 vs 16/55; Fisher's exact test, $p < 0.001$).

We also utilized the exome sequencing data to investigate DNA copy number alterations (Supplementary methods), both genome-wide (Supplementary Fig. 4 and Supplementary Table 6) and focusing on individual genes (Fig. 1) known to be commonly altered in prostate cancer [15]. Among these genes, the most frequently altered in our dataset were *NKX3-1*, *RB1*, and *CDKN1B*, with alterations in 24%, 25%, and 12% of the samples, respectively. Interestingly, the majority of the DNA copy number alterations were

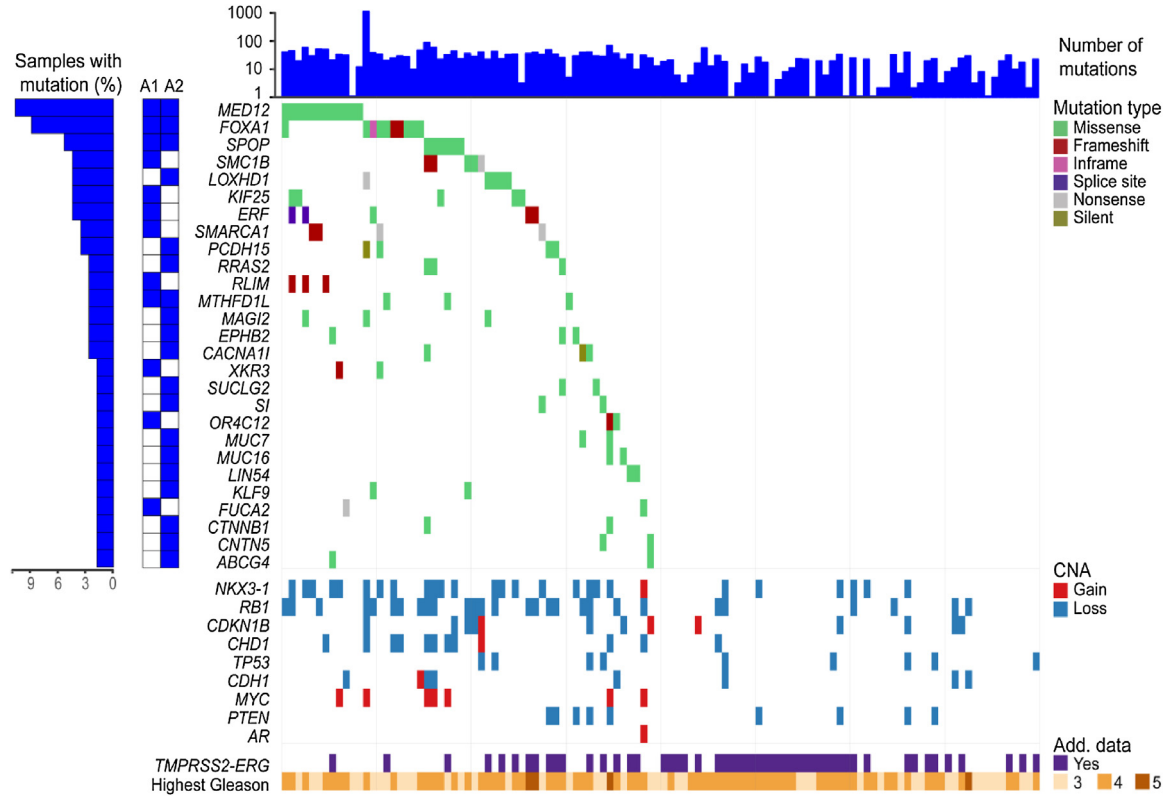


**Fig. 1 – Top-scoring altered genes in multifocal prostate cancer.**
**Top, the number of somatic point mutations identified in each of the 112 tumor samples. Center, top-scoring mutated genes from two algorithms, MutSig (A1) and Oncodrive-fm (A2), where each row represents a gene. From left, percentage of samples with mutation in the particular gene; blue squares indicate which algorithm(s) that identified the gene among its top 20 hits; Gene symbol; Mutations per sample, colored according to type. Bottom, presence of DNA copy number alterations in genes previously established to be commonly altered in prostate cancer. Additional data colored based on type; the most frequently found fusion gene in individual samples, *TMPRSS2-ERG*, and the highest Gleason grade associated with the tissue sample.**

found in the same samples that are mutated in our top-scoring genes. In fact, there is a strong correlation between number of mutations and percentage of genome with DNA copy number aberrations (Supplementary Fig. 5).

### 3.2. *Large degree of interfocal heterogeneity*

We next investigated the interfocal heterogeneity among the 89 studied tumor foci and observed unique sets of genes being altered in each individual tumor focus. In fact, when investigating the interfocal heterogeneity of all 1971 mutated genes, samples from different tumor foci within the same patient had on average less than one mutation in common. Among the top-scoring genes (Fig. 1), only two were mutated at the same site in more than one tumor focus of any patient (Fig. 2), reflecting a high degree of heterogeneity.

The 89 investigated tumor foci were from 41 patients, with two tumor foci from 34 patients and three tumor foci from seven patients (Table 1). This gives a total of 55 possible pairwise comparisons between tumor foci ($34 \times 1 + 7 \times 3 = 55$). The interfocal heterogeneity was large as 42 of the 55 comparisons (76%) revealed no common mutations (Fig. 3). In fact, 63 of the 89 tumor foci (71%) did not have any mutations in common with other tumor foci within the same patient. These 63 tumor foci were found in 30 of the 41 patients, meaning that 73% of the patients had at least one tumor focus with no mutations in common with the other tumor focus/foci in the same prostate (Supplementary Fig. 6).

The high degree of interfocal heterogeneity was also observed for DNA copy number alterations (Fig. 2). That is, genes commonly altered by DNA copy number alterations in prostate cancer, were only exceptionally affected in multiple tumors within the same prostate. Also, when performing unsupervised hierarchical clustering of DNA copy number data, samples from different foci within the same patient did not tend to cluster together (Supplementary Fig. 4).

To further strengthen the investigation of interfocal heterogeneity and to ensure that common mutations are not removed during the filtering process, we applied an additional, highly sensitive, mutation calling strategy with particularly loose filters (see Supplementary methods for details). To avoid scoring false-positive mutations, a given mutation identified with this sensitive method was only considered if it was also identified with the standard and more stringent analysis settings from another tumor sample within the same patient (Supplementary Fig. 7). The average number of common mutations within samples from different tumor foci was still only 1.5.

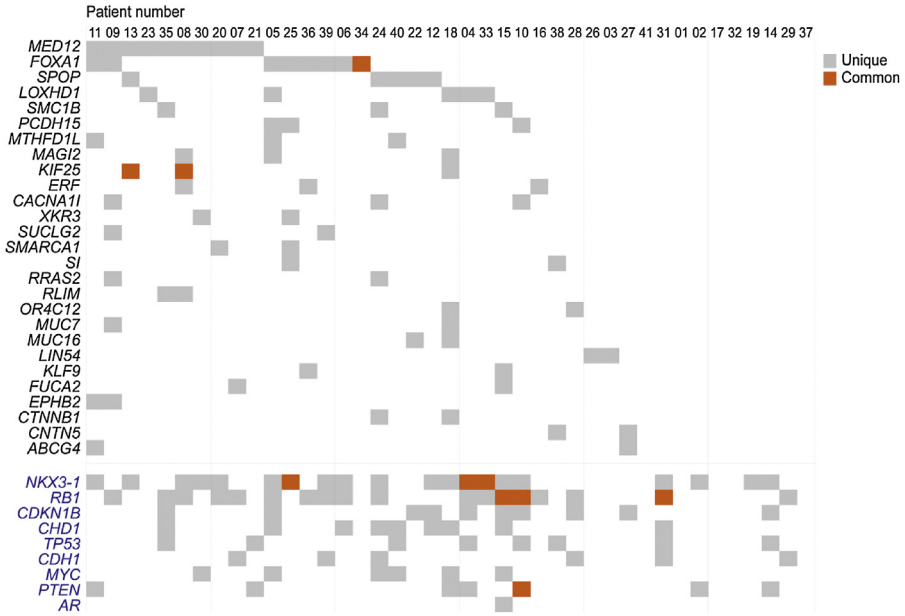


**Fig. 2 – The majority of mutations are not shared among cancer foci within the same patient.**
**Top: the top-scoring point mutated genes within each prostate. Bottom: genes commonly altered by DNA copy number aberrations. Each column represents a patient and each row a gene. Orange: a point mutation/DNA copy number alteration present in more than one tumor focus within a patient (common), grey: a point mutation/DNA copy number alteration only identified in one tumor focus within a patient (unique).**

### 3.3. *Substantial intrafocal heterogeneity*

Next, we investigated the intrafocal heterogeneity within 23 of our 89 tumor foci. For these foci, we sequenced two samples per focus and were able to compare the mutational patterns within a tumor. There was an average of 13 common mutations within the sample pairs (range, 0–36), and a median of 12 mutations was uniquely found in one of the two samples (range, 1–1378, or 1–75 when excluding the hypermutated sample).

### 3.4. *Common point mutations are seldom in known cancer-critical genes*

We next investigated whether the genes found to be commonly mutated across different tumor foci are enriched among the 594 cancer-critical genes in the Cancer Gene Census (Supplementary Table 7 [19]). While the overall list of mutated genes ($n = 1971$) is significantly enriched (94/594; $p < 0.001$), this was not the case for the genes with identical mutations in multiple tumor foci from the same prostate (standard mutation calling: 1/11, $p = 0.3$; high-sensitivity analysis: 2/46, $p = 0.7$).

## 4. Discussion

This study provides the first large-scale characterization of heterogeneity in somatic mutations among tumors from multifocal prostate cancer by using high-coverage whole-exome sequencing. To the best of our knowledge, this is the first time such a study has been performed on clearly distinct tumor foci, not just different areas of the same tumor, and within such a large number of patients. We unequivocally establish that the vast majority of tumor foci in the same prostate gland are independent tumors, without a shared precursor, and without shared mutational profiles. These results firmly emphasize complete interfocal genetic heterogeneity as the most common presentation in prostate cancer, rather than a rare, uncommon occurrence. These findings have major implications for how personalized prostate cancer medicine can be implemented, including the inability of a single tumor biopsy, or even multiple biopsies from the same focus to predict the clinical outcome in a given patient. This is important because the genotype, and thus phenotype, of one analyzed focus will not give the necessary information about other foci. Therefore, patients who are candidates, for example, for focal treatment, should have a thorough biopsying of their prostates before treatment decision is made to exclude the presence of other foci.

Using our mutation detection pipeline on the investigated clinical series, we revealed comparable mutational frequencies with previously published papers which included a single tumor per patient. Also, the significance analyses identified both known and novel mutated genes, confirming that our approach detects mutations relevant for the prostate tumorigenesis. *MED12*, *FOXA1*, and *SPOP* were found among the most frequently mutated genes in our study. Interestingly, however, these genes were seldom mutated in the same sample, tumor focus, or within tumors from the same patient, reflecting the high degree of interfocal heterogeneity we observed throughout the study.

Samples with expression of the fusion gene *TMPRSS2-ERG* had commonly none, and in general, few

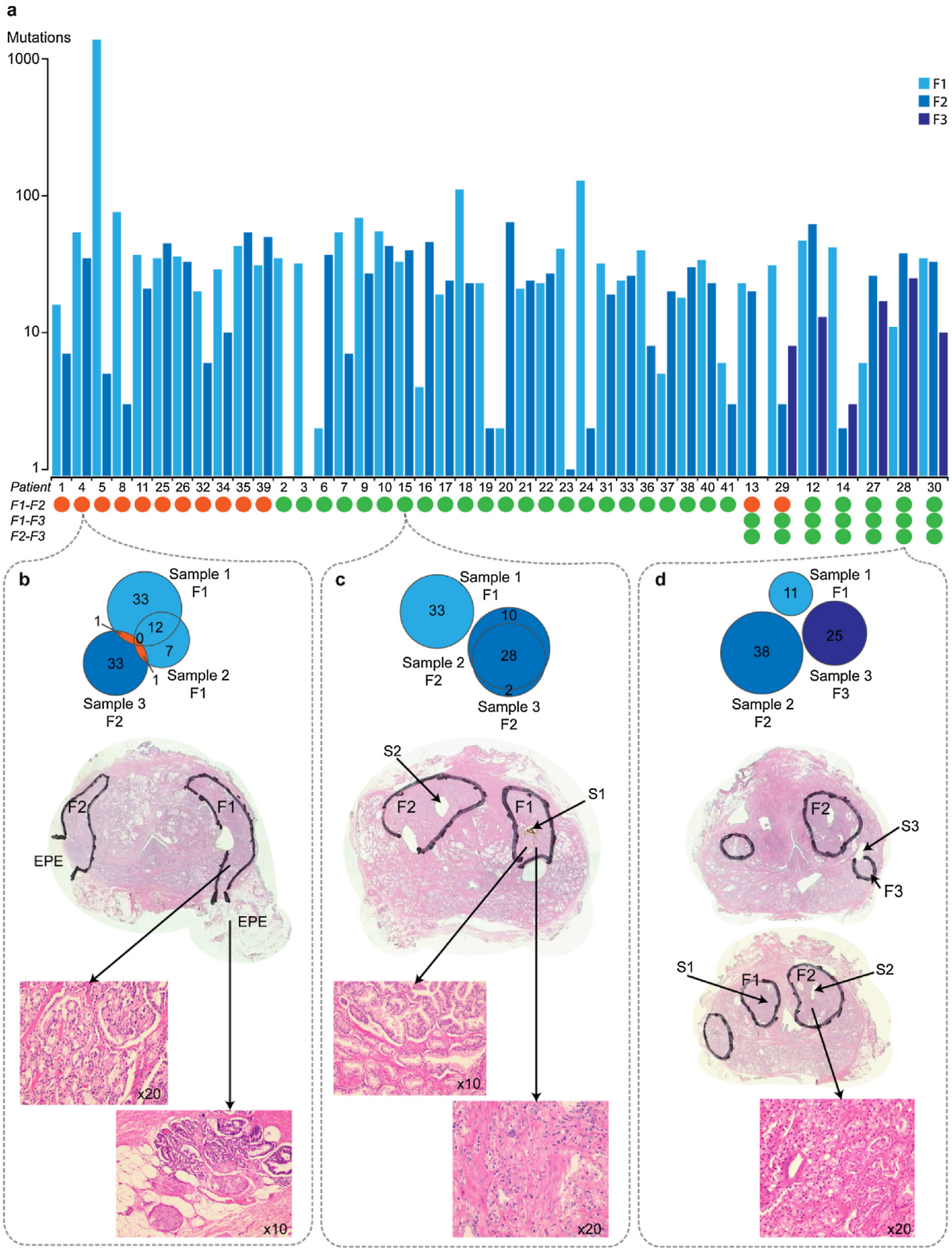


**Fig. 3 – Pairwise comparison of point mutations within distinct tumor foci.**
**(A) Number of point mutations identified within the 85 tumor foci investigated. Patients are sorted from left to right based on number of foci and the presence or absence of common mutations. Red circles: common mutations identified from the compared tumor foci; green circles: no common mutations identified from the compared tumor foci. (B–D) Example patients for which Venn diagrams show the number of mutations identified in each sample and how they are distributed among the tumor foci (top) and hematoxylin and eosin (HE) stains of representative tissue sections (bottom). (B) Patient 4. Samples 1 and 2 are both from focus 1. Two mutations are shared among the tumor foci. Both tumor foci show extraprostatic extension (EPE) and magnifications show Gleason score 4 + 4 = 8 (×20) and EPE (×10). (C) Patient 15. Samples 2 and 3 are from the same tumor focus. No common mutation is found in the foci. HE stained tissue section shows the location of samples 1 (S1) and 2 (S2) within tumor foci 1 and 2,**

somatic mutations in our top-scoring genes compared with samples not expressing the fusion gene. This is in concordance with a recent pan-cancer publication showing a significant decrease in mutational burden in cancer samples with fusions in driver genes, such as *TMPRSS2-ERG*, and might indicate that this fusion provides a strong enough enhancer of tumorigenesis to drive the cancer development with fewer other mutations [20].

Some previous studies have investigated the heterogeneity within multifocal prostate cancer, but with only two to four patients each [13–16]. In addition, multiple samples in these studies are selected from different areas of prostate tumors, not necessarily from different tumor foci and, thus, are not suited to investigate the interfocal heterogeneity. The present results, from clearly separated tumor foci, indicate that the different tumors within a prostate are very different from one another and most likely, the cancer progression is under the control of different driver genes in the individual tumors. This large degree of interfocal heterogeneity is also observed with the highly sensitive filtering of mutations, indicating that the observed heterogeneity is not caused by applying too strict filtering. Therefore, it is necessary to have information about all tumor foci to make a precise primary diagnosis, good prediction of the patient's prognosis, and best choice of treatment. This is not a trivial task; however, recent developments in use of liquid biopsies bear promise [21,22].

The here-observed interfocal heterogeneity is also interesting in light of recently published papers on metastatic prostate cancer [23–25]. Here, it has been shown that prostate cancer metastases have a monoclonal origin and a low degree of heterogeneity, indicating that they derive from the same clone in the primary tumor. Future studies, pinpointing which primary tumor foci that actually give rise to the metastases, are therefore highly warranted and may have great impact on future treatment decision-making.

This study also reveals a large degree of intrafocal heterogeneity, reflecting a continuous accumulation of mutations during the tumor growth or that what appears to be one tumor focus, is in fact the result of two or more merged tumor foci.

Finally, we observe that mutations found in multiple tumors within the same prostate only rarely affect known cancer-critical genes. This may be surprising and leads to speculation that majority of mutations in genes considered to be cancer-driving occur as later events unique to the particular tumor focus and often even unique to different areas within the same tumor focus.

## 5. Conclusions

By high-coverage whole-exome sequencing of multifocal prostate cancers, we have for the first time quantified the extent and frequency of the interfocal heterogeneity in primary prostate cancer. Both with regards to point mutations and DNA copy number alterations, the same alteration is rarely found in multiple tumors within the same prostate. Knowledge about this large degree of heterogeneity is essential for the management of prostate cancer as information from one tumor focus cannot be transferred to other foci present.

***Author contributions:*** Rolf Inge Skotheim had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

*Study concept and design:* K. Axcrona, Lothe, Skotheim.
*Acquisition of data:* Løvf, U. Axcrona, Bakken, Carm, Hoff, Myklebost, Meza-Zepeda, Lie.
*Analysis and interpretation of data:* Løvf, U. Axcrona, Zhao, Johannessen, Carm, Hoff, K. Axcrona, Lothe, Skotheim.
*Drafting of the manuscript:* Løvf, Zhao.
*Critical revision of the manuscript for important intellectual content:* Løvf, U. Axcrona, Zhao, Johannessen, Bakken, Carm, Hoff, Myklebost, Meza-Zepeda, Lie, K. Axcrona, Lothe, Skotheim.
*Statistical analysis:* Zhao, Johannessen.
*Obtaining funding:* Myklebost, K. Axcrona, Lothe, Skotheim.
*Administrative, technical, or material support:* U. Axcrona, Johannessen, Bakken, Meza-Zepeda, Lie, K. Axcrona.
*Supervision:* Løvf, Lothe, Skotheim.
*Other:* None.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at https://doi.org/10.1016/j.eururo.2018.08.009.

**respectively. Magnifications show representative Gleason score 4 + 3 = 7 (×10) and tertiary Gleason grade 5 (×20). (D) Patient 28. Three samples from three tumor foci. No common mutations identified. Two HE stained tissue sections show the location of the three samples within the three foci. Magnification shows region of the index tumor with Gleason score 4 + 5 = 9.**
**EPE = extraprostatic extension; F1 = focus 1; F2 = focus 2; F3 = focus 3; S1 = sample 1 location; S2 = sample 2 location; S3 = sample 3 location.**

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