

# Implications of Androgen Receptor Hyperstimulation by the FKBP51 L119P Mutation: No Evidence for Early Emergence of L119P in Prostate Cancer

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## Abstract

**Objective:** The immunophilin cochaperones, FKBP51 and FKBP52, have a modulating effect on steroid hormone receptors including the androgen receptor (AR). The differential effects seen by these immunophilins can be attributed to amino acid differences in the proline-rich loop; the proline at position 119 conferring AR potentiation capacity on FKBP52 and the leucine at this position in FKBP51 diminishing potentiation. FKBP51 can nevertheless potentiate AR activity in prostate cancer cells leading to accelerated growth. In addition, FKBP51 is regulated by AR, providing a feed-forward mechanism for FKBP51-mediated AR potentiation. These observations suggest that development of the FKBP51-L119P mutation in prostate cancer could lead to increased potentiation of AR and a more aggressive cancer phenotype. We tested this theory by examining the prevalence of FKBP51-L119P in a cohort of primary prostate tumours of increasing grade.

**Methods:** A segment of the FKBP51 gene containing the leucine 119 codon was amplified from tumour DNA by PCR then subjected to digestion with BsmAI, a restriction enzyme having a recognition sequence incorporating the leucine 119 'CTC' codon and occurring once in the amplicon. A subset of the prostate tumours was also examined for T > C conversion within the CTC codon by deep sequencing using the ion torrent system.

**Results:** We were unable to detect the L119P mutation in any of the prostate cancers examined by restriction-enzyme analysis irrespective of tumour grade. In addition we found no evidence from ion torrent sequencing of early clonal development of cells with the L119P mutation in a subset of the tumours.

**Conclusion:** We found no evidence to suggest that the L119P mutation contributes to an increasingly aggressive prostate cancer phenotype. However, tumour outgrowth favouring this mutation might occur in response to the low androgen environment and we propose examination for the L119P mutation in castrate-resistant prostate cancer.

**Keywords:** Androgen receptor; Prostate cancer; FKBP51 immunophilin; FKBP52 immunophilin; Proline-rich loop; Hyperactivating mutation; Restriction enzyme analysis; Ion torrent sequencing

## Introduction

The androgen receptor (AR) is the principal target of hormonal therapies in prostate cancer [1], but current therapies which exploit the dependence of AR on hormone activation become ineffective in castrate-resistant prostate cancer (CRPC), despite continued AR expression and evidence that androgen signalling is often maintained [2]. The unliganded AR is located primarily in the cytoplasm, assembled into a hormone-binding competent form with Hsp90 molecular chaperone machinery [3], which then facilitates efficient translocation of the hormone-bound AR-Hsp90 complex to the nucleus for transcriptional activation of target genes [4]. The mature AR complex consists of an Hsp90 dimer, p23 and one of the immunophilin cochaperones - FK506-binding proteins (FKBPs) 51 or 52 or cyclophilin 40 (CyP40), a binding protein for cyclosporin A (CsA) [5].

Although FKBP51 and FKBP52 (encoded by the *FKBP5* and *FKBP4* genes, respectively) share 70% sequence similarity and display similar domain organisation and structural architecture [4], mouse gene knockout strategies have revealed FKBP52, but not FKBP51 to be an important facilitator of steroid receptor activity in general [6-9]. These regulatory differences arise from FKBP52-mediated increases in receptor hormone-binding affinity leading to more efficient transcriptional activity, whereas competitive displacement of FKBP52 by FKBP51 acts to repress hormone binding, resulting in an attenuated hormonal response [6,7,10-12]. On the other hand, FKBP51 and FKBP52 are both positive regulators of AR-mediated prostate cancer cell growth [13-18] and may be targeted by FK506 to inhibit androgen-induced cell proliferation [13,17]. Furthermore, they may have relevance to androgen ablation therapy where reduced androgen levels are still effective in stimulating the receptor [15].

The stimulatory effect of FKBP52 is directed through the AR ligand-binding domain and recent studies have led to the proposal that a proline-rich loop, overhanging the FK1 catalytic pocket in FKBP52, makes specific contact with a region of the AR ligand-binding domain, most likely the AR BF-3 site, stabilizing a conformation optimal for high affinity hormone binding and efficient transcriptional activation [19-23]. Interestingly, the BF-3 surface of AR is known to harbour a large number of mutations involved in prostate cancer and androgen insensitivity syndrome [21,23,24]. FKBP52 is much more highly expressed in prostate cancer cell lines in comparison to normal prostate epithelial cells [13] and is upregulated in prostate cancer needle biopsies [14].

The FKBP51 gene is also emerging as a potential target of androgen signalling in the prostate with elevated levels of this cochaperone being observed in prostate cancers and FKBP51 overexpression able to stimulate prostate cancer cell growth [13,17,18]. In addition, FKBP51 was shown to severely affect the efficacy of bicalutamide, an antiandrogen used in patients undergoing androgen ablation therapy [18]. Moreover, in studies employing xenograft animal models, CWR22-R androgen-independent tumours that arose from androgen-dependent tumours (CWR22) expressed higher levels of FKBP51 [16,25]. FKBP51 expression decreased in the CWR22-R tumours of mice following androgen-ablation, only for levels to normalize over

time, and to elevate even higher in mice exposed to androgens [16], a pattern highly suggestive of a direct role for FKBP51 in prostate cancer growth and progression to the highly invasive androgen-independent state. These findings, along with additional experimental observations [26], demonstrate that FKBP51 is an androgen-regulated protein involved in an auto-regulatory pathway that increases androgen sensitivity.

The proline-rich loop (FKBP52 sequence: <sup>116</sup>AGS<sup>119</sup>PPKIP<sup>124</sup>P) is largely responsible for the functional difference between FKBP52 and FKBP51 relating to AR potentiation and repression of hormone binding, respectively [19]. The corresponding FKBP51 sequence (<sup>116</sup>AGS<sup>119</sup>LPKIP<sup>124</sup>S) differs at residues 119 and 124. However, the conformational changes in the proline-rich loop of FKBP51/52 that allow potentiation of AR activity are largely governed by amino acid residue 119 as proline substitution for Leu119 alone in FKBP51 converts the immunophilin to a potentiator of AR transcriptional activity mimicking that for FKBP52 [19]. Since the *FKBP5* gene encoding FKBP51 is a highly sensitive AR-regulated gene that is itself responsive to androgens [16,26] and increases AR activity in prostate cancer cells [18], such a gain-of-function mutant raises the possibility that this FKBP51 mutation results in increased androgen responsiveness, providing a selective growth advantage for affected prostatic tumours. Furthermore, a *FKBP5-ERG* fusion and a unique triple fusion (*TMPRSS2-FKBP5-ERG*) have been detected in primary prostate cancer tissue [27] suggesting a role for FKBP51 in prostate cancer development even prior to the selective pressures imposed by androgen deprivation therapy.

The aim of this study was to determine the underlying incidence of the FKBP51 L119P mutation in prostate cancer in a large cohort of prostate cancer samples of variable grade and to see whether presence of the mutation correlated with higher tumour grade.

## Materials and Methods

### Patients, patient samples and tumour grading

Prostate tissues were derived from radical prostatectomy (or biopsy specimens) from 50 Australian males. Tissue sections from three archival prostate cancer samples fixed in Solufix™ (each with matching normal prostate tissue sections) and a further 42 fresh frozen prostate samples were sourced from the UroPath Pathology Laboratory, West Leederville, Western Australia. An additional five fresh frozen tumours with matching normal prostate tissue were obtained from the Australian Prostate Cancer BioResource (APCB Victorian node, Monash University, Clayton, Victoria). Tumours were graded according to the Gleason scoring system [28] and allocated a pathologic stage according to the TNM system [29]. N-staging of regional lymph nodes was performed where appropriate. Tumour grading and pathologic staging of the tumours used in the study are shown in Table 1.

Informed consent was obtained from all patients for release of their tissue samples for experimental purposes: for UroPath samples, through the Hollywood Private Hospital Research Ethics Committee (approval number HPH128/230) and for APCB samples, through the Monash University Human Research Ethics Committee (approval number 1647). Experimental protocols were approved by the Sir Charles Gairdner Hospital Research Ethics Committee (Approval number 2009-051) and all experiments were performed in accordance with the relevant guidelines and regulations.

Gleason Score	Number of Samples	Pathological stage	Number of Samples
Benign hypertrophy	1	Benign hypertrophy	1
High grade dysplasia	1	High grade dysplasia	1
3+3=6	2	T2a	2
3+4=7 (4=20%)	3	T2c	10
3+4=7 (4=30%)	1	T2cNO	3
3+4=7 (4=40%)	5	T2cNX	1
4+3=7 (4=60%)	6	T3a	15
4+3=7 (4=70%)	3	T3aNO	9
4+3=7 (4=80%)	8	T3b	3
4+4=8	10	T3bNO	1
4+5=9	9	T3bN1	4
5+4=9	1		

**Table 1:** Tumour grading and pathological staging of the fifty prostate tumours/prostatic tissues used for assessing the prevalence of the L119P mutation.

### Cell culture

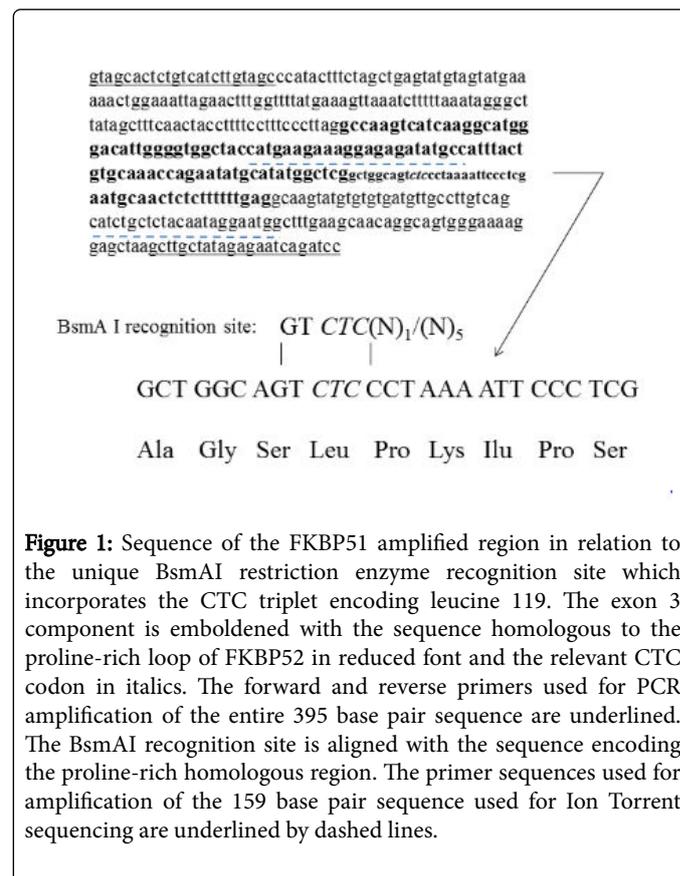
The AR negative cell lines DU145 and PC-3 and the AR positive cell lines LNCaP, C4-2B (a metastatic derivative of LNCaP), 22RV1 and PC-3-AR (an AR-transfected derivative of PC-3) were propagated in DMEM medium containing 5% fetal bovine serum (FBS) (or 10% FBS in the case of LNCaP cells) and penicillin/streptomycin and incubated in 5% CO<sub>2</sub> at 37°C as described previously [30].

### DNA extractions

Fresh frozen samples (15-25 mg of tissue in a partially frozen state) were sliced using a sterile scalpel blade into thin sections and placed in an Eppendorf tube on ice. DNA was recovered from the prepared tissue samples by proteinase K digestion followed by binding and elution of the DNA from a mini spin column using the QIAamp DNA Mini Kit (Qiagen, Venlo, Limburg, Netherlands) according to the manufacturer's instructions. For the archival material, eight 10 µm sections were dewaxed in xylene (3 x 30 min) then chloroform (30 min), and rehydrated in absolute ethanol (30 min), 75% ethanol (30 min) and PBS (2 x 30 min) prior to DNA extraction, which was performed in a similar manner to that for fresh frozen samples except that proteinase K concentrations were increased 2-3 fold. DNA was eluted from spin columns with 100 µl or 150 µl of sterile ddH<sub>2</sub>O for the archival and fresh frozen tissue samples, respectively. DNA yields ranged from 1.5-9 ng/µl for the archival tissue to 20-600 ng/µl for the fresh frozen samples. Cell culture monolayers grown to near confluency were trypsinised and DNA was extracted from 3 x 10<sup>6</sup> cells following proteinase K digestion using the QIAamp DNA Mini Kit. DNA was eluted with 200 µl of sterile ddH<sub>2</sub>O yielding concentrations of 120-376 ng/µl.

### PCR and PCR product purification

A 395 bp fragment of the FKBP51 gene containing all of exon 3 including codon 119 (CTC) was amplified from prostate DNA using a KAPA HiFi PCR kit (Kapa Biosystems, Wilmington, MA, USA) with intronic forward, 5'-GTAGCACTCTGTCATCTTGTAGC-3', and reverse, 5'-GGATCTGAT'TCTCTATAGCAAGC-3', primers (Figure 1).



**Figure 1:** Sequence of the FKBP51 amplified region in relation to the unique BsmAI restriction enzyme recognition site which incorporates the CTC triplet encoding leucine 119. The exon 3 component is emboldened with the sequence homologous to the proline-rich loop of FKBP52 in reduced font and the relevant CTC codon in italics. The forward and reverse primers used for PCR amplification of the entire 395 base pair sequence are underlined. The BsmAI recognition site is aligned with the sequence encoding the proline-rich homologous region. The primer sequences used for amplification of the 159 base pair sequence used for Ion Torrent sequencing are underlined by dashed lines.

Reaction volumes of 25 µl included 1X KAPA HiFi Fidelity buffer, 2 mM MgCl<sub>2</sub>, 0.3 mM dNTPs, 0.3 µM each primer, 0.5 U KAPA HiFi DNA polymerase and 10-50 ng template DNA. Cycling conditions were as follows: 95°C/5 min, 35 cycles of 98°C/20 sec, 60°C/15 sec, 72°C/15 sec then 72°C/5 min. For archival samples where PCR product yields were low, reactions performed in duplicate or triplicate were combined for analysis. The 395 bp FKBP51 fragment was similarly amplified from DNA extracted from prostate cancer cell lines using Go Taq Flexi DNA polymerase (Promega Corp, Madison, WI, USA) with the above mentioned primers. Reaction volumes of 50 µl included 1X Go Taq Flexi buffer, 2 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.4 µM each primer, 1.5 U polymerase and 250-500 ng template DNA. Cycling conditions were as follows: 94°C/270 sec, 35 cycles of 94°C/45 sec, 60°C/45 sec, 72°C/1 min then 72°C/10 min. Amplified PCR products were purified using a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions, with DNA yields ranging from 25-75 ng/µl for archival tissues, 75-200 ng/µl for fresh frozen tumour material and 38-46 ng/µl for cell lines.

### Restriction enzyme digestion

Purified FKBP51 PCR amplicons from prostate cancer cell lines, prostate tissue and controls were digested with BsmAI (isoschizomers

Alw26I and BcoDI; New England Biolabs, Ipswich, MA, USA). The BsmAI restriction enzyme site (GTCTC(N)<sub>1</sub>/(N)<sub>5</sub>) is located once in the 395 bp sequence and encompasses the leucine 119 codon (Figure 1) with complete digestion generating 250 and 145 bp fragments. Digestions (20 µl) containing 1X cut smart buffer and 7.5 U BsmAI enzyme and 300-368 ng DNA (cell lines) or 225-500 ng DNA (prostate tissue) were incubated at 55°C for 4-6 hours (2 hours for cell lines) with intermittent mixing and centrifugation to ensure complete digestion. Reactions were similarly incubated for a further 2-4 hours following addition of an extra 5 U enzyme and digestion products were separated in 1.5% agarose gels.

### Deep sequencing using the ion torrent system

A 159 bp fragment of the FKBP51 gene containing codon 119 was amplified from three archival and five fresh frozen prostate cancer DNA samples and their matching normal prostate tissue (Table 2) using AmpliTaq Gold 360 DNA polymerase (Invitrogen Life Technologies) with the exonic forward primer 5'-C ATGAAGAAAGGAGAGATATGCC-3' and the intronic reverse primer 5'-CCATTCCTATTGTAGAGCAGATG-3' (Figure 1). Reactions (25 µl) contained 1X AmpliTaq Gold 360 DNA buffer, 2 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.5 µM each primer, 0.625 U AmpliTaq Gold 360 DNA

polymerase and 25 ng template DNA. Cycling conditions were as follows: 95°C/10 min, 30 cycles of 95°C/30 sec, 55°C/30 sec, 72°C/30 sec then 72°C/7 min. Ion Torrent sequencing (using the Ion One Touch 200 template kit) and bioinformatics analyses were undertaken at the Australian Genome Research Facility, Brisbane (University of Queensland) and Melbourne (Walter and Eliza Hall Institute) nodes, respectively. For ion torrent sequencing, primers were generated in which the forward exonic FKBP51 primer above was fused to the Ion A adapter sequence as well as to a unique barcode sequence to identify each sample. The reverse FKBP51 intronic primer was fused to the Ion Torrent specific P1 adapter sequence to create the reverse fusion primer. Using the 159 bp amplicons as template and the above-described fusion primers, adapter-ligated libraries were amplified onto beads which were then centrifuged into wells on a CMOS semiconductor microchip [31]. Single nucleotide variant reads were performed using Partek GS algorithms (Partek Genomics Suite software: Version - Revision 6.6 Build 6.13.0731; Copyright 2014 Partek Inc., St Louis, MO, USA). Total reads for the 16 samples ranged from 12301-23532 (Table 2) and the mean read length ranged from 111-124 base pairs. We examined the percentage of total reads assigned to each nucleotide at the middle position of the CTC codon (a T to C conversion signifying a leucine to proline substitution).

Patient number	Samples	Total Reads	Nucleotide at T position (% of total reads)			
			T	G	C	A
70081 (ff)	Benign	16342	99.566	0	0.428	0.006
	Malignant (GS: 3+3=6)	23532	99.427	0.013	0.532	0.03
70090 (ff)	Benign	19294	99.337	0	0.643	0.021
	Malignant (GS: 3+3=6)	22131	99.517	0.005	0.465	0.014
70080 (ff)	Benign	17476	99.336	0.011	0.635	0.017
	Malignant (GS: 4+3=7)	16460	99.453	0	0.529	0.018
70602 (ff)	Benign	12301	99.228	0	0.74	0.033
	Malignant (GS: 4+4=8)	14785	99.357	0.014	0.582	0.047
70309 (ff)	Benign	15569	99.416	0	0.565	0.019
	Malignant (GS: 4+5=9)	15228	99.422	0.007	0.565	0.007
2617G (archival)	Benign	15591	98.576	0	1.411	0.013
	Malignant (GS: 3+4=7)	13234	99.441	0.015	0.529	0.015
2424J (archival)	Benign	19016	99.27	0.011	0.694	0.026
	Malignant (GS: 4+3=7)	13449	99.546	0	0.446	0.007
2451K (archival)	Benign	14408	99.334	0.007	0.659	0
	Malignant (GS: 3+4=7)	14245	99.389	0	0.604	0.007

**Table 2:** Prevalence in benign and malignant prostatic tissue of a T to C change in CTC codon 119 leading to CTC->CCC. ff: fresh frozen; GS: Gleason score

## Results

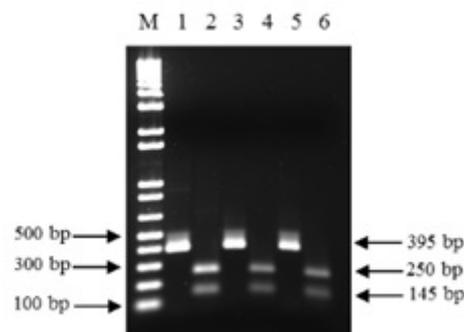
### Histopathological analysis of prostate tumours

The prevalence of mutations affecting codon 119 of FKBP51 was examined in 50 prostate tumour specimens 47 (94%) of which were from fresh frozen tumour samples with the remainder from archival formalin-fixed tissue. Forty-six of the samples were medium to high grade prostate tumours (Gleason scores >7), but there were two low grade tumours (Gleason score 6) and one sample each of high grade dysplasia (prostatic intraepithelial neoplasia) and benign prostatic hyperplasia (Table 1). Of the 48 tumours 46 (96%) were peripheral zone tumours the majority of which (83%) were associated with multifocal lower grade peripheral and/or transition zone tumours which were not assessed. Primary tumour volume ranged from 0.2-12.46 cc (average from 48 samples: 3.77 cc) and the percentage of tumour in each specimen was estimated to range from 80-100%. The pathological stage of frank tumors ranged from T2a to T3bN1 (Table 1). Extraprostatic extension of tumours (stage T3a and higher) was established in 32/50 (64%) of cases. One stage T3a tumour showed invasion of the left ejaculatory duct, however the left seminal vesicle was not represented in the tissue for confirmation of its involvement. Nine cases were stage T3b indicating definite spread to the seminal vesicles, either directly via the ejaculatory duct or through metastatic deposits; of these, four exhibited regional lymph node metastasis (T3bN1). There were no cases of stage IV tumours (spread beyond the seminal vesicles to other organs), although one tumour classified as T3a NO showed invasion of bladder neck parenchyma.

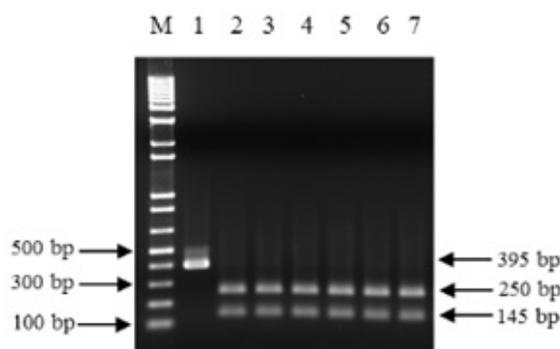
### Restriction enzyme analysis for the L119P mutation in prostate samples

Initial analyses using the on-line mutation prediction tool 'Mutation Taster' ([www.mutationtaster.org](http://www.mutationtaster.org)) and examination of the 1000 genomes browser (<http://browser.1000genomes.org>) indicated that *FKBP5* c.356T>C that converts the CTC codon to CCC is not a registered disease-causing sequence variant, nor is it an identified natural polymorphism.

For restriction enzyme analysis we took advantage of a BsmAI restriction enzyme site that encompasses the leucine 119 codon and is located once in the 395 bp PCR-generated, FKBP51-exon 3 sequence (Figure 1). PCR amplification of FKBP51 exon 3 followed by over digestion with BsmAI did not provide evidence of sequence alterations affecting the enzyme recognition site in any of the prostate tumour/prostate biopsy specimens, six prostate cancer cell lines or control specimens examined, i.e. all samples appeared to digest completely (Figures 2 and 3). In addition, direct sequence analysis of amplicon cell line DNA yielded wild type sequence in all cases (results not shown). Due to the relatively low sensitivity of this method, the findings indicated that, if present, FKBP51 codon 119 mutations represented a very small proportion of FKBP51 exon 3 sequence in the tissue or cell line DNA. In particular, the generation of tumour clones resulting from early mutational events in the tumours might be difficult to detect using this method.



**Figure 2:** No evidence for the presence of the L119P mutation in BsmAI digests from prostate cancer tumours. Ethidium bromide-stained 1.5% agarose gel image of PCR-amplified FKBP51 exon 3 DNA from a prostate tumour (Gleason score: 4+3=7) either undigested (lane 3) or digested with BsmAI (lane 4) and from a prostate tumour (Gleason score: 4+4=8) either undigested (lane 5) or digested with BsmAI (lane 6). Control undigested and digested DNA (extracted from the lymphocytes of a subject without prostate cancer) is shown in lanes 1 and 2, respectively (two-fold excess DNA was used for the control to ensure complete digestion). The image was generated on a ChemiDoc digital imager (BioRad) and is representative of several gels in which 50 prostate tumour digests were examined. M=Molecular weight marker with the positions of 100, 300 and 500 base pair (bp) bands indicated.



**Figure 3:** No evidence for the presence of the L119P mutation in BsmAI digests from prostate cancer cell lines. Ethidium bromide-stained 1.5% agarose gel image of PCR-amplified FKBP51 exon 3 DNA from undigested LNCaP prostate cancer cell line (lane 1) and BsmAI digested LNCaP, DU145, C4-2B, 22RV-1, PC-3 and PC-3-AR prostate cancer cell lines (lanes 2-7, respectively). The image was generated on a ChemiDoc digital imager (BioRad). M=Molecular weight marker with the positions of 100, 300 and 500 base pair (bp) bands indicated.

## Deep sequence analysis for the L119P mutation in prostate samples

In order to detect possible early development of the L119P mutation we performed deep sequencing of DNA extracted from eight of the prostate tumours as well as their matched nonmalignant tissue controls. This analysis showed that at position 356 of the coding region, thymine was the predominant nucleotide in all samples scoring over 99% of total reads in most cases and while cytosine was more common than either adenine or guanine, it generally represented <1% of total reads (Table 2). The only exception was the matched benign prostatic tissue from a patient with a low grade prostate cancer where cytosine represented 1.4% of total reads. In general, there was no appreciable difference in the prevalence of cytosine either between samples when comparing all the malignant or benign tissues or within a particular patient's matched samples, when comparing benign and malignant tissue. In fact, in most cases the benign tissue scored equally or higher than the corresponding malignant tissue. The overall higher prevalence of cytosine compared with adenine or guanine at position 356 is unlikely to indicate the presence of a true 'C' allele as the percentage value for 'C' is still within the normal error incorporation rate for ion torrent sequencing and may reflect its position within a cytosine polymer (Figure 1), homopolymers reportedly leading to a greater error rate in ion torrent sequencing [32,33]. In summary, these data do not support the selective clonal development of mutations at the 'T' site in the Leu119 codon in low grade or more advanced primary prostatic tumours, however the possibility that this mutation is selected for in CRPC patients in response to a low androgen milieu remains to be investigated.

## Discussion

FKBP51 has the capacity to potentiate AR-mediated prostate cell growth and the *FKBP5* gene is itself androgen regulated [13,16-18,26]. These observations, coupled with experiments demonstrating that the FKBP51 L119P mutation restores AR potentiation to that demonstrated by FKBP52 [19], have led to the hypothesis that the L119P mutation might occur naturally in the evolution of prostate cancers leading to accentuated potentiation and a more aggressive prostate cancer phenotype. In order to test the prevalence of this mutation in prostate cancer we examined approximately 50 prostate tumours representing a broad range of Gleason score and pathological staging, as well as several AR-dependent and AR independent prostate cancer cell lines. Tumour and cell line DNA was examined for the mutation by conventional means (PCR amplification and restriction enzyme analysis) in all cases and a subset of the tumours with their matched normal prostate tissue was also examined by deep sequencing. In addition, we examined by restriction enzyme analysis a further 39 prostate cancer samples that, although processed for RNA, still contained amplifiable amounts of DNA (results not shown); these tumours also covered a wide range of Gleason score (Gleason 5-9, with the majority (67%) being Gleason 7) and pathological staging ranging from T2c-T3bN1. We found no evidence for the presence of the L119P mutation by restriction enzyme analysis in any of the prostate cancers including those tumours of higher grade and the prostate cancer cell lines also did not harbour this mutation. There was no evidence from deep sequencing of early clonal development of cells with the L119P mutation in a subset of the tumours. In view of these findings, this study approach could be extended to examine other potentially activating mutations in FKBP51, for example in regions of the FK1 domain such as the  $\beta$ 3 bulge where sequence divergence between

FKBP51 and FKBP52 leads to conformational changes that favour FKBP52-mediated AR potentiation [22].

Our study examined only solid, primary prostate tumours following prostatectomy. Tumours were generally confined to the prostate gland or had limited extraprostatic extension. Extensive metastatic disease was not examined, nor was local or metastatic recurrence following androgen deprivation therapy (ADT) i.e. CRPC. It is possible that the L119P mutation arises selectively in response to the low androgen milieu following ADT and therefore would have been missed in this study. Indeed, recent investigations have demonstrated that >70% of CRPC cases harbor genetic alterations affecting the AR pathway and while most of these involve the AR itself, other AR signaling factors may also be affected [34,35]. Mechanisms by which CRPCs adapt to the androgen depleted environment have been reviewed by Ferraldeschi et al [36]. Although most mechanisms focus directly on the AR or on androgen signalling, alterations in AR cochaperones might also provide a growth advantage for prostate cancer cells following ADT. While proposed candidates include p23 and FKBP52 [6,9,13,20,37], it is now clear that FKBP51 may also be important [16-18], for example FKBP51 is upregulated in LAPC-4 tumours grown in mice under androgen deprivation and stimulates AR-mediated transcription and prostate cell growth [18]. Such activity, coupled with the introduction of an FKBP51 L119P hyperactivating mutation could provide a highly effective mechanism for the prostate cancer cell to compensate for the androgen-depleted environment and underscores the need to investigate the presence of this mutation in metastatic tumour tissue (eg from bone, lung or lymph nodes) in patients with CRPC. Finally, since metastatic tumour material is often difficult to obtain, there is a growing trend to examining circulating tumour cells (CTCs) for the presence of mutations [38] and this approach could be adopted to investigate the presence of the L119P mutation in CRPC patients. Extensive whole exome and/or transcriptome sequencing of CTCs or, alternatively, re-evaluation of bioinformatical data already generated [34] may yet reveal the presence of the L119P mutation in a subset of CRPCs.

Prostate cancer displays extensive genetic heterogeneity. For any given patient, prostate cancer is frequently multifocal with foci of disease of separate clonal origin arising independently and characterized by unique genetic alterations (interfocal heterogeneity). Even within a particular focus of disease, cancer cells may exhibit differences in genomic alterations (intrafocal heterogeneity) (reviewed by Boyd et al [35]). Most of the tumours we examined were multifocal with only the largest focus selected for genetic analysis. The FKBP51 L119P mutation may therefore have been present in other foci that were not selected. In addition, it was estimated that samples were composed of 80-100% tumour tissue leaving the potential for up to 20% of the tissue being stroma. Since only a proportion of the frozen tissue (approximately 1/10<sup>th</sup>) was used for DNA extraction it is possible that the L119P mutation may have been missed if stroma tissue was largely included or if the primary focus was characterized by extensive intrafocal heterogeneity. The use of laser capture microdissection could have aided in the identification of single prostate tumour cells for use in genetic analysis [39]. The lack of detection in the prostate cancer cell lines is perhaps not surprising as other genetic defects have been attributed to their cancer phenotypes [35,40]; we confirm that the FKBP51 L119P mutation does not contribute to these cancer phenotypes.

The genetic susceptibility to prostate cancer and the landscape of genetic alterations in both primary prostate cancer and CRPC have

been evaluated over recent years with inactivation of tumour suppressor genes (eg *PTEN* or *TP53*), chromosomal rearrangements (eg ETS oncogene fusions such as *TMPRSS2-ERG*) and alterations in AR signaling genes (eg *AR*, *FOXA1*, *SPOP*) amongst the genetic changes commonly detected [34,35,41-45]. To our knowledge there are no SNPs or polymorphisms linking prostate cancer susceptibility to the *FKBP5* gene, nor have somatic mutations or gene copy number alterations been detected in the *FKBP5* gene associated with either primary prostate cancer or CRPC. However, a *FKBP5-ERG* fusion and a unique triple fusion (*TMPRSS2-FKBP5-ERG*) have been uncovered by next generation transcriptome sequencing of primary prostate cancer tissue [27] and it was considered that the triple fusion in particular might confer a growth advantage to the cell in a low androgen environment. In addition, mutations in the *CTNNB1* gene detected in CRPC [34] may have implications for  $\beta$ -catenin's synergistic action with FKBP52 in potentiating hormone-independent AR signaling [46]. Furthermore, c-Myc, which is commonly amplified in prostate cancers [35], positively regulates FKBP52 expression [47] and the progressive loss during prostate cancer progression of the AR regulatory protein, ID4, potentiates FKBP52-AR interaction, thus promoting a CRPC phenotype [48]. These observations and a recent study demonstrating the selective advantage of *FKBP4* gene amplification and/or overexpression in driving castration resistance [49] provide strong evidence of a role for the FKBP immunophilins in the development of CRPC.

In summary, we were unable to detect the FKBP51 L119P mutation in a cohort of primary, predominantly non-invasive, prostate cancers of variable Gleason score and pathological grade using restriction enzyme analysis, and deep sequencing of a proportion of the tumours did not reveal clonal outgrowths containing the mutation. Therefore, there is no evidence to suggest that the FKBP51 L119P mutation contributes to the development of a more aggressive prostate cancer phenotype at least for primary tumours. However, taking advantage of new technologies facilitating the mutational examination of CTCs, attention should be drawn to assessing the prevalence of this mutation in CRPC as the FKBP51 L119P mutation may be particularly associated with this phenotype as the cell attempts to evade or overcome the low androgen environment.

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