Expression of Phosphorylated-mTOR During the Development of Prostate Cancer

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BACKGROUND. The PI3K pathway plays a significant role in the progression of prostate cancer (PCa) to an advanced stage. Mouse models suggest that the downstream effector molecule of the PI3K pathway, mTOR, is also important in the development of PCa, where it plays a pivotal role in forming precursor lesions such as high grade prostatic intraepithelial neoplasia (HGPIN). This study was conducted to determine the status of phosphorylated-mTOR (p-mTOR the activated state of mTOR) across the PCa progression model by looking at expression in normal prostate tissue, proliferative inflammatory atrophy (PIA), HGPIN, and PCa.

METHODS. Expression of p-mTOR was evaluated by immunohistochemistry on tissue microarrays constructed from 120 archival formalin-fixed paraffin embedded radical prostatectomy tissue specimens. Levels of expression were recorded as the percentage of positive epithelial cells multiplied by the intensity of staining scored as 0–3.

RESULTS. p-mTOR expression was found to increase across the progression model with mean staining in non-neoplastic samples of 40 compared to 98 in PIA, 107 in HGPIN, and 136 in cancer ($P < 0.001$), but without significant increase between HGPIN and PIA. Correlation of high p-mTOR expression with outcome in PCa showed a trend towards worse prognosis, but this was not statistically significant.

CONCLUSIONS. This study demonstrates that p-mTOR signaling has a potential role in both the initiation and progression of PCa. These data provide support for further research into the possible use of rapamycin analogues in the treatment of PCa, and raise the possibility that mTOR might be a potential target for chemoprevention. *Prostate 74:1231–1239, 2014.*

KEY WORDS: **prostate; carcinoma; mTOR; prostatic intraepithelial neoplasia; atrophy**

INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed cancer in the US accounting for an estimated 238,590 new cancer cases, 28% of cancer in males, and 29,720 deaths per year [1]. Prostate cancer is also a leading cause of cancer deaths in males in other developed Western countries and causes significant morbidity and mortality globally. Understanding the

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biology responsible for prostate carcinogenesis and progression has the potential to improve cancer care through the identification of molecular markers that stratify patients at diagnosis into different risk categories enabling better targeting of therapy, and ultimately through the development of novel chemoprevention strategies.

Precursor lesions allow us to study the early molecular changes that initiate the development of PCa. The best-studied pre-invasive lesion is high-grade prostatic intraepithelial neoplasia (HGPIN), which is the neoplastic transformation of the epithelial lining of the prostatic ducts and acini without invasion through the basement membrane [2]. De Marzo et al. [3], postulated that another lesion, proliferative inflammatory atrophy (PIA), is a precursor to HGPIN; they defined this lesion as a discrete focus of proliferative glandular epithelium with the morphological appearance of simple atrophy or post-atrophic hyperplasia, occurring in association with inflammation, and often morphologically merging with HGPIN [3]. PIA exhibits an increased Ki-67 proliferation rate and has been found to share some of the same molecular and chromosomal changes as HGPIN, such as increased expression of cMET and cEPBβ [4], hypermethylation of *GSTP1* CpG islands, chromosome 8 changes, and mutations in p53 and AR alleles [5,6].

The PI3K/AKT/mTOR pathway has long been known to play an important role in the development of PCa [7]; PTEN is a tumor suppressor gene that inhibits the activation of this pathway through inhibition of PI3K. Furthermore, PTEN mutations and deletions have been detected in 60% of metastatic PCa, and 20% of localized PCa [8]. Loss of PTEN in the mouse prostate leads to the development of murine HGPIN followed by invasive adenocarcinoma and metastatic disease, which like human prostate cancers responds to castration [9]. PTEN loss leads to activation of AKT and its downstream targets and thus AKT is also likely to be involved in PCa development. This hypothesis is supported by mouse models such as the TRAMP mouse model of PCa, in which increased expression of PI3K and phospho-AKT correlate with the development of PIN-like lesions followed by adenocarcinoma and then metastatic disease [10]. The postulated role of AKT signaling in the development of PCa is further supported by the MPAKT mouse, which has murine prostate restricted Akt kinase activity resulting in HGPIN-like lesions [11]. Moreover, the development of these HGPIN lesions in the MPAKT mouse can be abrogated by mTOR inhibition [12], a downstream target of phospho-AKT. This suggests that mTOR is essential for the downstream effect of the PI3K/AKT pathway in prostate carcinogenesis.

mTOR signaling is a therapeutically attractive target as there are commercially available inhibitors of mTOR already in use in the treatment of renal cell cancers and breast cancers. To date there are conflicting data on the expression of mTOR in PCa and HGPIN; there have only been small cohorts of HGPIN lesions examined with differing results [13–16]. In this study with a larger cohort of precursor lesions, we aim to investigate whether phosphorylated-mTOR (p-mTOR) expression changes early in PCa development by assessing its expression across non-neoplastic tissue, PIA, HGPIN and PCa.

METHODS

Patients

Tissue for the TMAs was taken from a cohort of radical prostatectomy specimens obtained from the archives of the Department of Anatomical Pathology (RP) at St. Vincent's Hospital and Douglass Hanly Moir Pathology (Sydney, New South Wales, Australia) between 1987 and 2000. All surgery was performed by one of six specialist urologists. Patients who received neoadjuvant hormonal therapy were excluded from the study. Patients were followed post-operatively by their surgeons on a monthly basis until satisfactory urinary continence was obtained and then at 3-month intervals until the end of the first year, at 6-monthly intervals to 5 years and yearly thereafter. Relapse was defined by the following criteria: biochemical disease progression with a serum PSA concentration $\geq$0.2 ng/ml increasing over a 3-month period or local recurrence on digital rectal examination confirmed by biopsy or by subsequent rise in PSA. Clinical relapse was defined as local recurrence or distant metastatic disease. These patients are part of a group of well-characterized patients with a diagnosis of localized PCa who underwent RP as their primary treatment [17] and who on pathological review, contained the lesions of interest, and represent those patients for whom FFPE tissue was available at the time of the study. This project was approved by the St. Vincent's Campus Human Research Ethics Committee (Reference number: H00/088).

Tissue Microarrays

Medium density TMAs were constructed using the MTA1 tissue arrayer (Beecher Instruments, Silver Spring, MD). For each case up to four, 1 mm cores of PCa, PIN, PIA, or non-neoplastic tissue were arrayed. Hematoxylin and eosin-stained sections of the donor blocks were used by a urological pathologist (JGK) to identify the lesions. Non-neoplastic tissue was selected

from areas greater than 3 mm away from other lesions and PIN and cancer were marked according to accepted criteria [18]. Proliferative inflammatory atrophy was defined as simple atrophy or post atrophic hyperplasia associated with inflammation as per De Marzo et al. [3].

Four-micrometer sections were cut from the constructed array blocks. Verification of pathology was performed on each tissue core by a histopathologist (JGK) from a TMA section stained with hematoxylin and eosin. Total number of cores grouped by lesion are listed in Table I.

Immunohistochemistry

p-mTOR expression was studied using immunohistochemistry on consecutive sections of the TMAs. Four-micrometer sections of these specimens were cut, mounted on Superfrost Plus adhesion slides (Lomb Scientific, Sydney, Australia) and heated in a convention oven at 75°C for 2 hr to promote adherence to the slide. Sections were dewaxed and rehydrated before antigen-retrieval. Antigen retrieval was performed in a pressure cooker for 60 sec at pH 6.0. TMAs were stained with primary antibody against p-mTOR (Cell Signaling Technology, Beverely, MA; *#9559*, *#2976*) at 1:50 for 30 min at room temperature. p-mTOR was detected with DAKO EnVision Rabbit (DAKO Corporation, Carpinteria, CA) and substrate liquid 3,3′-diaminobenzidine Plus (DAKO Corporation). Counterstaining was performed with hematoxylin. Staining specificity was assessed in negative controls, by substituting the primary antibody with rabbit IgG (RIgG, DAKO Corporation) of the same concentration and a second negative control using antibody incubated with p-mTOR blocking peptide (Cell Signaling Technology; *#1230*). Positive controls were breast cancer cell lines, MCF-7, and MDA MB-231 that express p-mTOR when grown in normal growth medium [19].

Immunostaining was scored by microscopic assessment of the percentage of lesional cells with positive cytoplasmic staining. Staining intensity was graded between 0 and 3, with 0 representing no staining, 1 weak, 2 moderate, and 3 strong intensity staining (Fig. 1). Immunostaining for p-mTOR was assessed independently by two observers (SIMS and JGK) who were blinded to outcome. Significant discrepancies were resolved by consensus on a multi-viewer microscope. A final immunohistochemical score was determined by multiplying the highest lesional cytoplasmic intensity of staining by the mean percentage of positive cells stained to yield a value between 0 and 300 for each case represented on the TMAs.

TABLE I. TMA Lesions Analyzed

	Number of patients (cores)
Total	120
Non-neoplasia	67 (105)
Atrophy (non-inflammatory)	63 (107)
PIA	90 (215)
HGPIN	81 (162)
Cancer	103 (265)
Gleason pattern	
G2	8 (8)
G3	83 (165)
G4	48 (81)
G5	5 (8)

Statistical Analyses

The significance of differences in immunostaining between the pathologies was analyzed using ANOVA and independent *t*-tests. Disease-specific relapse was measured from the date of RP to the date of relapse or last follow-up. Correlation of immunostaining with biochemical relapse was done using a Kaplan–Meier survival curve and a Log Rank test for significance in a stepwise fashion (i.e., using a cut off of 50, then 100 up to 250) to reveal the natural split of the data [17]. All data were analyzed using SPSS v21 (SPSS, Inc., Chicago, IL). Statistical significance in this study was set at $P < 0.05$.

RESULTS

p-mTOR expression was assessed in 854 cores from 120 patients in lesions representing cancer (265 cores), HGPIN (162), PIA (215), and non-neoplastic tissue (105) (Table I). In these cores, p-mTOR staining was observed in the cytoplasm of the epithelial cells (Figs. 1 and 2). In non-neoplastic glands staining was noted in the cytoplasm of basal epithelial cells but this was not recorded in scoring the percentage of cells staining positive. Low grade PIN was observed to have greater intensity of staining than non-neoplasia, but the number of lesions ($n = 3$) were too low to be included in the analysis.

Changes in PIA, HGPIN, and Cancer

p-mTOR expression was increased across the PCa progression model (Fig. 3), with mean immunostaining in non-neoplastic samples of 40 (95% CI 27–52) compared to a mean of 98.0 in PIA (95% CI 86–110), 107 in HGPIN (95% CI 93–121), and 136.0

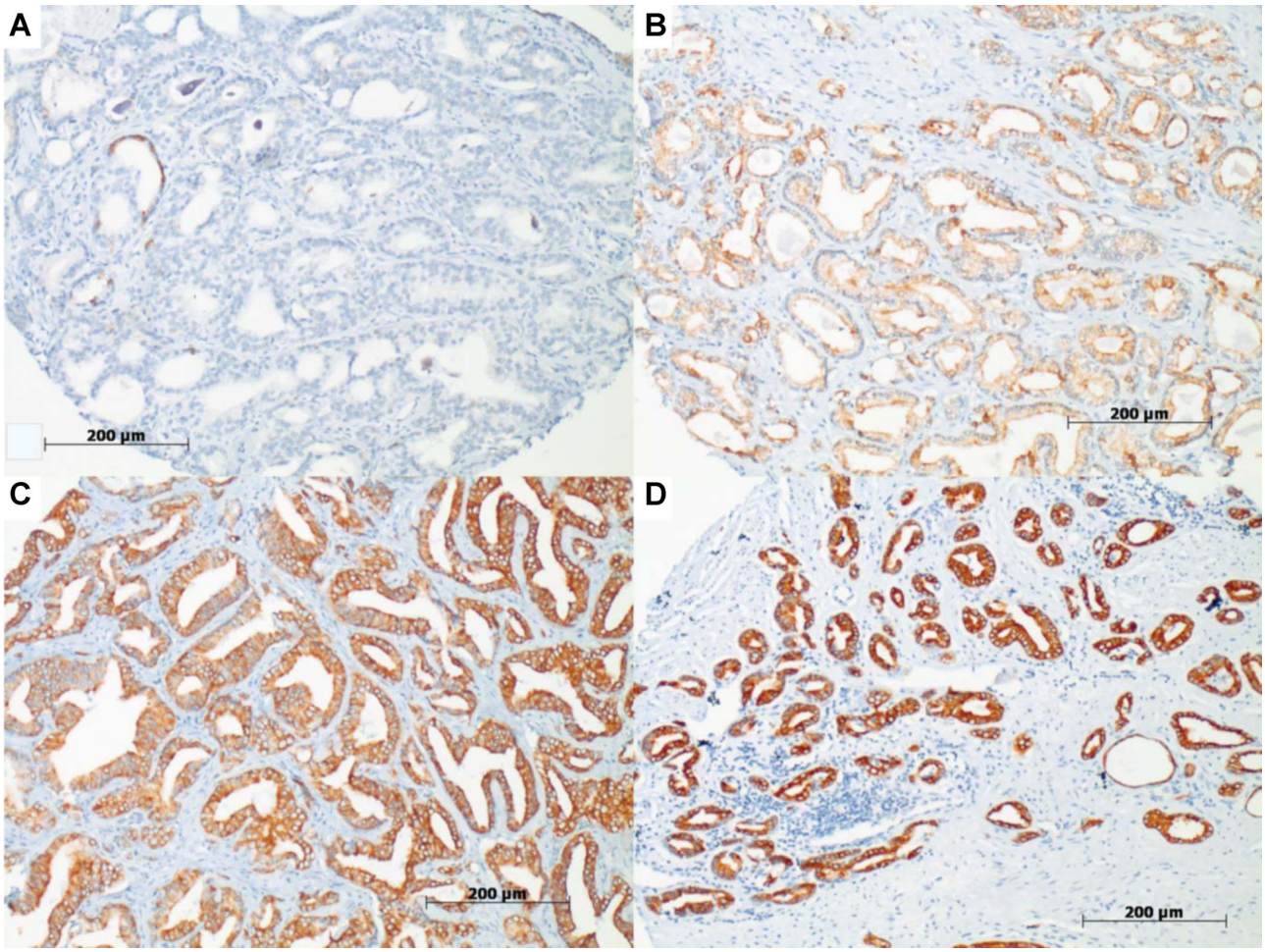

Fig. 1. Photomicrographs of p-mTOR immunostaining in prostate cancer. **A**: No staining. **B**: 1+ intensity staining. **C**: 2+ staining. **D**: 3+ staining (original magnification 100×).

in cancer (95% CI 118–155). ANOVA testing showed that the means were significantly different ($P < 0.001$). An independent samples *t*-test confirmed that the mean of p-mTOR expression in cancer, HGPIN, and PIA was significantly higher than that in non-neoplastic tissue with $P < 0.001$ in all three tests and mean p-mTOR expression in cancer was higher than that of HGPIN ($P = 0.023$) and PIA ($P < 0.001$). Using an independent sample *t*-test, there was no difference in mean p-mTOR expression between HGPIN and PIA ($P = 0.35$). In the 77 patients with paired HGPIN and carcinoma, 36 individual cases had a higher immunohistochemical staining score in the carcinoma compared to the HGPIN (with an average increase in score of 119), 17 cases had a comparable level of staining, and 24 cases had a lower IHC score in the carcinoma than the HGPIN (with an average decrease of 108). These data demonstrate a statistically significant increase in p-mTOR expression from non-neoplastic prostate tissue through to pre-cancerous lesions and finally PCa.

Relationship With Clinicopathological Parameters

In the cohort of 103 cancer patients, there was a median follow-up of 12 years (range 8.5–16 years) with 42/103 (41%) having experienced biochemical relapse. There was a large variation in p-mTOR expression across the cohort (Fig. 4a). At a cut point of 100 for p-mTOR expression, 33% (15/45) of patients with p-mTOR expression <100 had relapsed compared to 47% (27/58) relapses in the higher scoring group, ≥100 ($P = 0.33$). Although, there was a trend towards a worse prognosis in those with higher p-mTOR expression, this was not statistically significant as the study was underpowered for this endpoint (Fig. 4b). The relationship between p-mTOR expression and other common clinicopathologic variables

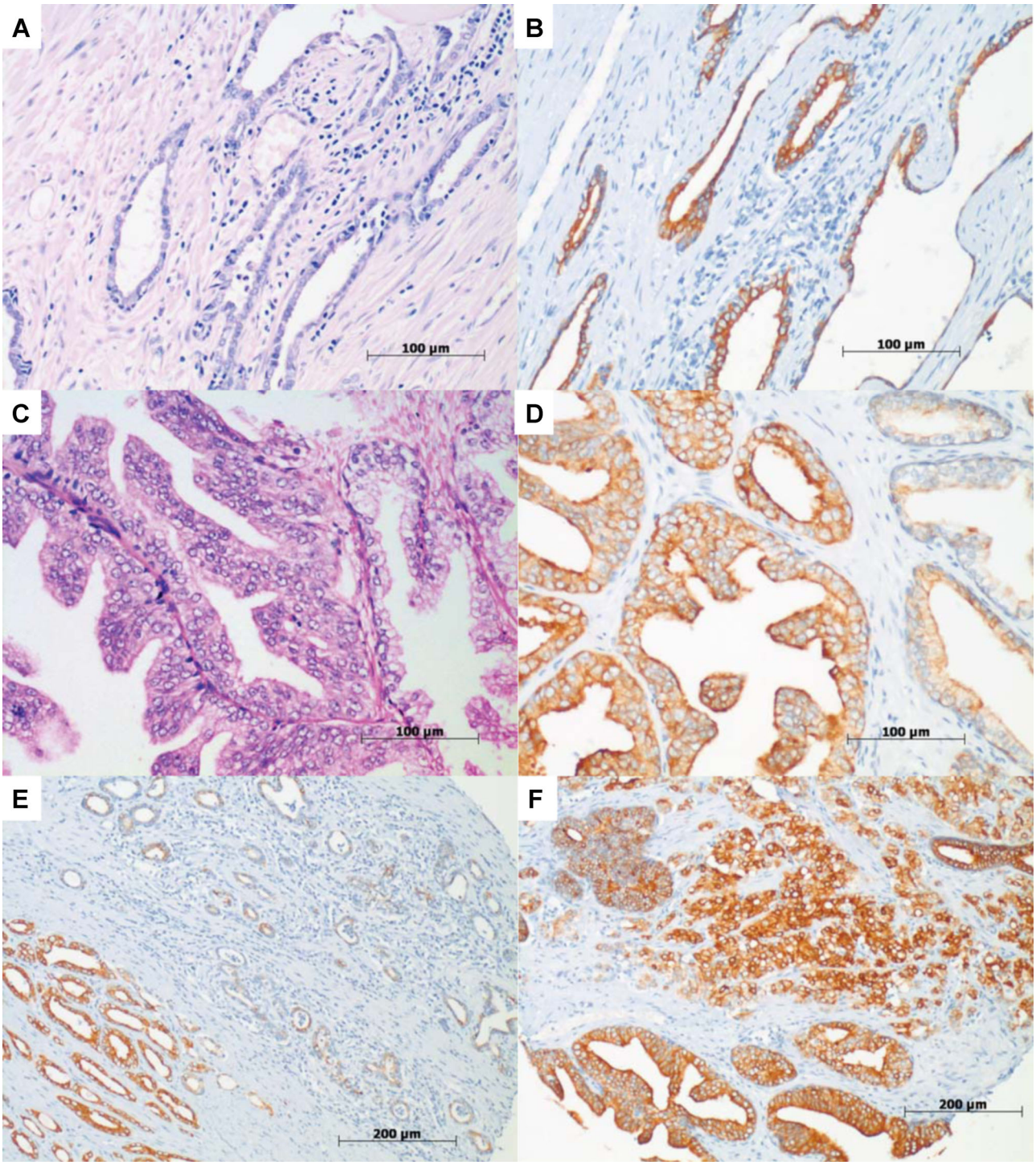

Fig. 2. **A**: Simple atrophy with associated inflammatory infiltrate (H&E stain, original magnification 200×). **B**: 3+ intensity staining for p-mTOR in PIA. **C**: HGPIN (left side) adjacent to non-neoplastic prostate tissue (right) (H&E stain, original magnification 200×). **D**: 2+ intensity staining for p-mTOR in HGPIN (left) and 1+ staining in adjacent non-neoplastic tissue (right). **E**: 3+ p-mTOR in Gleason pattern 3 cancer (bottom left) and 1+ in adjacent PIA (original magnification 100×). **F**: 3+ staining for p-mTOR in Gleason pattern 4 cancer (top) and adjacent HGPIN (original magnification 100×).

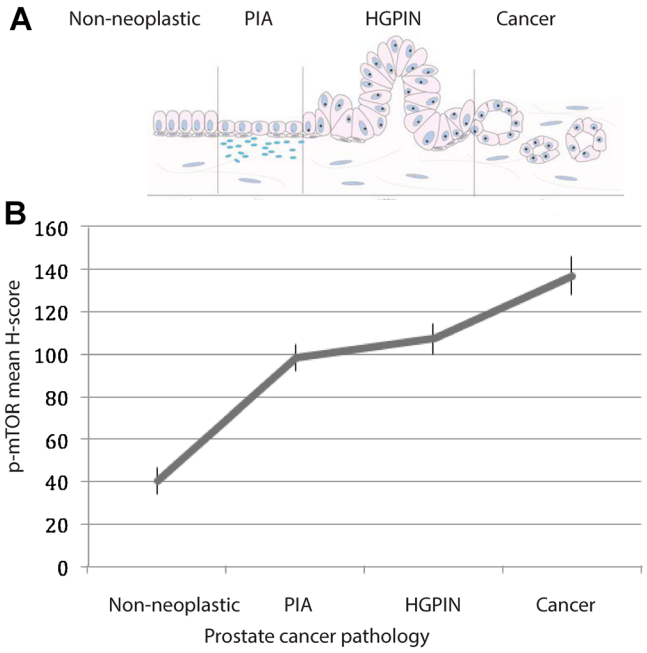

Fig. 3. **A**: Schematic presentation of the Prostate Cancer Progression Model demonstrating morphological changes associated with the development of Proliferative Inflammatory Atrophy (PIA), High Grade Prostatic Intraepithelial Neoplasia (HGPIN) and invasive carcinoma (Cancer). **B**: Mean p-mTOR expression across the prostate cancer progression model.

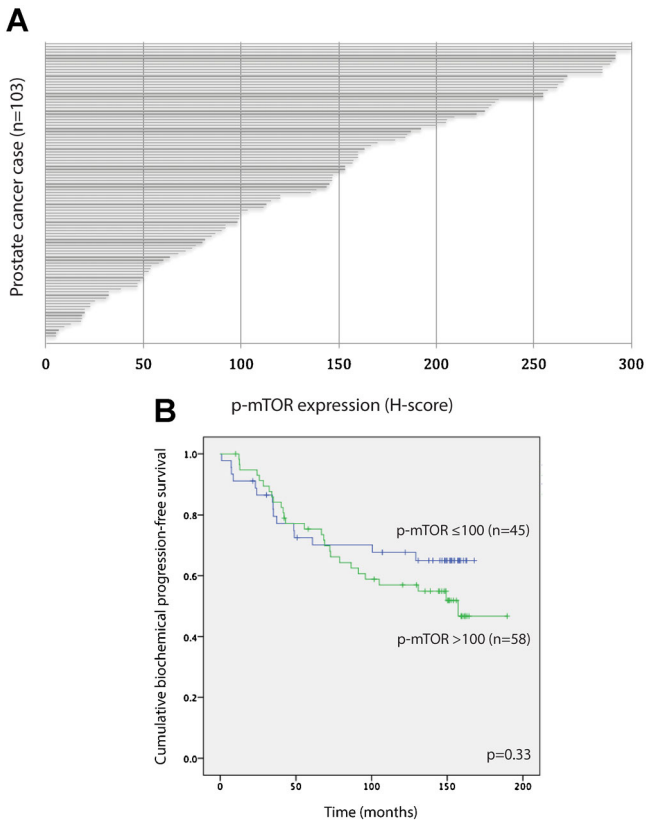

Fig. 4. **A**: Variation in p-mTOR expression across the prostate cancer cohort. **B**: Kaplan–Meier curve of progression free survival with high or lower p-mTOR expression (cut point 100).

was assessed, however, there were no significant correlations with pre-operative PSA, Gleason score and pathological stage (Table II).

DISCUSSION

In this study, we have shown that p-mTOR expression increases across the PCa progression model. Upregulation of p-mTOR expression in one premalignant lesion (HGPIN) compared to normal glands has been documented previously [13–16,20], and this study now shows this observation to be generalized across the spectrum of putative premalignant prostate lesions, showing that upregulation of p-mTOR is an early process and establishing it a potential driver of carcinogenesis of the prostate. This work raises the possibility that mTOR might be a potential target for chemoprevention.

Despite the murine data implicating mTOR signaling in prostate carcinogenesis [10–12], human studies of p-mTOR expression have produced conflicting results. In contrast to our study, of the four previous series [13–16], only Dai et al. [13] found upregulation of p-mTOR in cancer compared to HGPIN with increased staining in 53% of PCa (97/182) compared to 10% (1/10) in HGPIN and 5% (1/20) in BPH. Although, Dai et al. [13] utilized the same antibody at the same dilution (1:50), they used less intensive antigen retrieval (water bath) than our study (pressure cooker) probably accounting for the lower level of positive staining observed in HGPIN (1 of 10 cases) and carcinoma (58% with weak staining in <10% cells). The largest cohort of HGPIN evaluated prior to our study was reported by Evren et al. [14], who assessed 35 cases of HGPIN plus 144 cases of PCa observing reduced p-mTOR expression in cancer (only

TABLE II. Clinicopathologic Features and p-mTOR Expression

Clincopathologic feature	Number of patients	Mean staining score	*P* value
Stage			
Stage ≤T2	66	50	0.31
Stage ≥T3	37	56	
Gleason score			
≤6 or 7 = (3 + 4)	76	50	0.38
7 = (4 + 3) or ≥8	26	56	
Pre op PSA			
<10	64	48	0.54
≤10	28	22	

66% of cancer cases had increased staining, defined as positive staining in >5% of cells), compared to HGPIN (in which 83% of cases had increased levels of p-mTOR staining). Similarly, to our cohort Evren and colleagues used RP specimens with the HGPIN lesions coming from the same cohort of patients, however, the discordant findings could be attributable to the lesser number of HGPIN samples evaluated by their study (35 vs. 81), their cohort of patients comprising a higher grade/less differentiated group with 26% of carcinomas having a Gleason score of ≥8 (compared to 7% in ours), variation in immunohistochemical methods, and differences in IHC scoring systems. While Evren et al. [14] also used the same p-mTOR antibody as in the current study, antigen retrieval and incubation with the primary antibody were both longer. Moreover, staining was evaluated using an automated IHC scoring system (Aperio T3 Scan Scope), which, as opposed to looking at a spectrum of staining intensity, assessed the area of positive staining in each core (dichotomized at a low cut point of 5% of the core) [14]. This gave a much higher rate of positive staining in normal tissue but does not reflect whether the overall intensity of staining was less than in cancer or HGPIN. Taking these factors into account we believe our results are the most robust, especially given the much larger cohort evaluated (81 cases of HGPIN in our study vs. 10 in Dai et al., 12 in Kremer et al., and 35 in Evren et al.) [13,14,16].

p-mTOR is an important molecule in the PI3K/AKT pathway, which has been demonstrated to be involved in PCa development and progression. This pathway has been implicated in advanced PCa where loss of PTEN, the upstream molecule that suppresses this pathway, occurs in more than 20% of localized prostate cancers and 60% of metastatic disease and results in upregulation of PI3K and AKT [21]. It has also been proposed to play a role in castrate resistance [22] and the clinical activity of the mTOR inhibitor, everolimus, has been assessed in a small phase II clinical trial in combination with bicalutamide [23]. Mouse models, however, suggest that dysregulation of the PI3K/AKT pathway could be a much earlier event in the development of PCa, resulting in the development of the precursor lesion HGPIN [9–12]. In this study, we demonstrated in human prostate tissue that p-mTOR is not only upregulated in HGPIN, but also in the proposed precursor lesion PIA.

The molecular and genetic make-up of PIA has been examined, but not in the context of the PI3K/AKT pathway. Markers of proliferation such as Ki-67 and proliferative cell nuclear antigen are upregulated in PIA [4,24] as are levels of Bcl-2, an anti-apoptotic protein [25], while levels of cell cycle regulatory proteins such as $p27^{Kip1}$ and cyclin-dependent kinase inhibitor are reduced [6] and expression of p53, cMET, and cEBPβ are increased [4,26]. Interestingly, expression of p16, a cell cycle regulator protein, is increased and this is thought to be secondary to response to cellular stress [27]. It has been hypothesized that cell stress may play a role in the development of PIA as proteins involved in the stress response, including GSTP1, glutathione S-transferase A1, and COX-2, have been shown to be upregulated [6,24,26,28]. Interestingly, the protein NKX 3.1 is reduced in PIA, causing increased susceptibility to DNA damage from oxidative stress [29].

Studies of the early molecular changes in prostate carcinogenesis facilitate the identification of potential targets for chemoprevention. To date, 5-alpha reductase inhibitors are the most well studied chemoprevention strategy in PCa, with two large randomized control trials demonstrating a risk reduction between 23% and 25%. However, their uptake has been hindered by the concern that both these trials found an increase in high-grade PCa in the treatment arm. Epidemiology studies have suggested that dietary interventions could also reduce the risk of PCa but trials of dietary interventions such as The Selenium and Vitamin E Cancer Prevention Trial (SELECT), did not prevent the development of PCa in healthy men [30]. Thus to date, there is still scope for an effective chemopreventative strategy, and perhaps, a strategy that targets a higher risk population group. All of these trials have looked at prevention in either healthy men or men with an elevated PSA, however, we know that the risk of PCa increases by over 2–4 fold in those individuals with a first degree relative with the disease [31,32]; thus, investigating primary prevention in this group of patients may be a more effective strategy.

This is the first study to look at the association between p-mTOR expression and PCa biochemical relapse. There was a non-significant trend towards higher p-mTOR expression predicting for poorer prognosis. This result does not support the finding by Dai et al. 2009 that p-mTOR expression decreases with increased Gleason score, a known poor prognostic factor, however, other studies have shown that genomic deletion of PTEN as well as reduced expression of PTEN, an inhibitor of the PI3K/AKT pathway, are associated with early biochemical recurrence [33–35]. Other investigators have examined the predictive power of loss of PTEN expression in combination with increased expression of pAKT and Gleason score [36], PTEN loss combined with p27 [37] loss, and loss PTEN combined with gain of c-MYC copy number [38]. In all of these studies PTEN loss was found to associated with a higher risk of biochemical recurrence. Hence,

our findings warrant further investigation in a larger cohort of men with localized PCa.

CONCLUSION

Our study has demonstrated that p-mTOR upregulation occurs early in the development of PCa and that expression of p-mTOR is increased in the putative precursor lesion PIA, providing new evidence for PIA's potential role in prostate carcinogenesis. In addition, these findings raise the possibility that p-mTOR might be a potential target for chemoprevention in high-risk patients although further research is necessary to determine the feasibility of this approach.

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