Original Article

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# An Immunohistochemical Study for Mammalian Target of Rapamycin (mTOR) Signaling Pathway Including Interacting PTEN in Prostatic Acinar Adenocarcinoma and Correlation with the Patient Clinicopathological Parameters

**Somaia Ahmed Saad El-Din[1,2] , Shaima Abdel Moety[3], Khalid Al Hashmi[4], Shadia Al Sinawi[3], Suaad Al-Badi[3], Afrah Al Rashdi[3], Samya Al Husaini[3], Hajer Albadi[3] and Asem Shalaby[5]**

[1]Pathology Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt

[2]Department of Histopathology, Armed Forces Hospital, Muscat, Oman

[3]Pathology Department, Sultan Qaboos University Hospital, Muscat, Oman

[4]Hematology and Medical Oncology Department, Armed Forces Hospital, Muscat, Oman

[5]Pathology Department, Mansoura University, Mansoura, Egypt

## Abstract

**Background**: The activation of AKT-mTOR-PTEN pathway may promote prostate cancer progression and affects response to targeted therapies. The full extent of this activation remains to be determined. Our aim: was to assess the expression of inactive mTOR, phosphor-mTOR, phosphor-AKT and loss of PTEN in prostatic adenocarcinomas then correlate their expression with the clinicopathological parameters.

**Methods**: The study included 166 prostatic adenocarcinoma tissues using immunohistochemistry on tissue microarrays. Statistical analysis considering markers expression and correlation with the clinicopathologic parameters was done using appropriate tests.

**Result**: The mean age was 72.63 and 75.9% were clinically high risk. Gleason score 7 and WHO grade group 5 were the commonest (31.3% and 31.9% respectively). Most patient (73.1%) were stage T2 or higher. Expression of inactive mTOR, phospho-mTOR and phosphor-AKT was seen in 96.1%, 93.5% and 95.9% respectively. The loss of PTEN expression was noted in 55.3%. There were significant correlations between Gleason pattern 4 and the expression of inactive mTOR (p value <0.001 and 0.004 respectively) and phospho-mTOR (p value 0.003 and 0.001 respectively). Gleason score 7 was significantly correlated to inactive mTOR expression (p value <0.001). There was also significant correlation between phosphor-AKT and phospho- mTOR expression with p value 0.004.

**Conclusion**: The immunohistochemical expression of inactive mTOR, phosphor-mTOR and phosphor-AKT and loss of PTEN was appreciated in most prostate cancer cases, suggesting that activation of this pathway occur early during prostate tumorigenesis. This may indicate that targeting mTOR pathway may have a promising therapeutic role in the management of prostatic adenocarcinoma.

***Keywords**: Prostate tumorigenesis, mTOR, AKT, PTEN, Therapeutic target, prostatic adenocarcinoma*

## Introduction

Worldwide, prostate cancer is the second most common malignancy among men and the sixth leading cause of death [1]. In Oman, prostate cancer is the most frequent cancer among men, accounting for 10.29%, followed by colorectal cancer (9.32%) according to the cancer incidence registry (2016) [2].

According to the SEER (Surveillance, Epidemiology, and End Results) database, the prognosis of patients with localized or regional disease is good with 5 years survival rate reaching 99%, but that is not the case for patients with metastatic disease who usually have a worse prognosis with low 5-year survival rate mostly not exceeding 31% [3].

The androgen deprivation therapy through either chemical

or surgical castration is currently considered as the first-line treatment for metastatic disease but the response is temporary and many patients usually progress to castration-resistant status (CRPC) [4]. Thus, there is a strong need to find different therapeutic targets for those patients.

The phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway has been strongly implicated in carcinogenesis in a wide variety of tumor types including prostate cancer, with mTOR inhibitors, proved highly successful in prolonging free survival in breast and renal cancer [5]. Moreover, it is also reported that inhibition of androgen receptor (AR) leads to subsequent activation of PI3K/AKT/mTOR thus enabling the prostate cancer cell to survive and progress [6]. This may explain the rationale to use inhibitors of Pi3K/AKT/mTOR pathway in such situation. Also, activation of AKT/mTOR pathway may help in chemotherapy and radiation resistance in prostate cancer [7].

AKT, is a serine-threonine protein kinase, that is broadly expressed in most tissues and organs, and is activated through phosphorylation by phosphoinositide 3-kinase at residue Ser 473 [8]. The activation of AKT results in activation of mTOR at serine residue 2448 (S2448) [9] and this activation results in the phosphorylation of downstream effectors, such as eukaryotic initiation factor 4E (eIF4E) and the ribosomal S6 kinase 1 (S6K1). On the other hand, PTEN (phosphatase and tensin homolog deleted on chromosome 10), a well-recognized tumor suppressor gene, is a negative regulator of AKT. The loss of PTEN expression leads to activation of PI3K- AKT- mTOR signaling pathway [10,11]. Up to date, there are conflicting results for p-mTOR activation pathway in prostatic adenocarcinoma. Stello et al [12] reported that activation of mTOR pathway is associated with favorable outcome. However, Shorning et al [5] found that activation of mTOR signaling pathway is associated with disease progression and therapeutic resistance.

Here, we investigated the immunohistochemical expression of phosphorylated AKT 473, inactive mTOR, phosphorylated mTOR (S2448),) as well as PTEN in prostate adenocarcinoma and correlated this with different clinicopathological parameters as tumor differentiation, stage and progression in attempt to find a rational for using the mTOR pathway inhibitors in management of patients with this tumor especially those with advanced prostate cancer as they have limited therapeutic options. Moreover, we investigated the possibility of using these markers to distinguish indolent from aggressive disease in patients with localized tumors.

## Material and methods

### *Patient samples*

The present study was approved by The Ethics Committee of College of Medicine and Health Sciences, Sultan Qaboos University (MREC#256/2020) and by the Ethics Committee in Armed Forces Hospital (AFH), Muscat, Oman. A total of 166 male patients with prostatic adenocarcinoma were enrolled in the present study. The inclusion criteria for cases selection were cases from January 2007 to December 2018 that underwent core needle biopsy, transurethral resection prostate (TURP) or prostatic surgical excision with available tissue blocks. Cases with no any available tissue blocks were excluded.

All available clinical information concerning patients' age, symptoms whether obstructive or irritative or others, signs (Digital rectal examination) for hard nodular suspicious prostate, initial serum PSA level, clinical risk group (that incorporate 3 grades of low, intermediate and high according to 3 parameters including local prostate examination, initial PSA level and Gleason Score/WHO grade group), patient general condition concerning kidney function test and liver function test (or any other general health problem that may affect the choice of therapy as congestive heart failure, or other cancer), tumor stage whether localized to prostate involving one or both lobes, or associated with bone or lymph node metastasis, therapy used whether hormonal, surgical, radiotherapy or chemotherapy and response to therapy (remission versus castrate resistant) through post treatment PSA serum level and tumor recurrence.

### *Tissue microarray (TMA) construction:*

Histological slides of all patients (n=166) were retrieved from the pathology archives together with corresponding paraffin blocks containing the largest tumor volume (with tumor of at least 0.5 cm in largest dimension per paraffin block). In addition, the following control specimens were selected: normal / non neoplastic prostate tissues (n=20), invasive ductal mammary adenocarcinomas (n=3), normal kidney (n=3), colon (n=3), placenta (n=3), tonsil (n=3), thyroid (n=3), testis (n=3) and proliferative endometrium (n=3).

For tissue microarray (TMA) construction, three cylindrical cores (diameter 1 mm) were taken from representative areas in the donor paraffin blocks for mostly each case and transferred to recipient paraffin blocks using Manual Tissue Arrayer MTA-1 from Estigen OÜ, Tiigi 61b, 50410 Tartu, Estonia. In total, six TMA blocks were constructed each including 90 specimens incorporating the prostate cancer cores along with control cores.

### *Immunohistochemistry and interpretation:*

Tissue slides (5 μm) were mounted on aminoacetylsilane-coated glass slides (Starfrost, Berlin, Germany), deparaffinized in xylene, washed in ethanol and rehydrated in water. The tissues were stained for the expression of inactive mTOR, phosphorylated mTOR (Ser2448) (p-mTOR), phosphorylated AKT (Ser473) (p-AKT) and PTEN using a standardized protocol on the Ventana Benchmark® Ultra system automatic monostainer (Ventana Medical

Systems). Details are provided in Table 1. The control tissue samples were examined first. Both the percentage of tumor cells with positive staining and the intensity of the stain was documented in semiquantitative way. The 3 tissue cores of each case were analyzed for most cases and the average score was used for statistical analysis. Finally, the stained slides were screened under a light microscope using the power × 20 and the staining intensity and distribution of each protein (inactive mTOR, p-mTOR, p-AKT and PTEN) were assessed semi-quantitatively in the neoplastic cells and the results were defined as; negative when no reactivity or weak positive reactivity in less than 5% of tumour cells, as low /patchy positive when positive reactivity affects more than 5% of tumour cells but less than 50% with weak to moderate stain intensity (score 1 to 2) and as high positive if more than 50% of the neoplastic cells show moderate or strong stain (score 2 or 3) [12].

The pattern of staining for all tested antibodies was cytoplasmic with or without nuclear staining. No cases showed nuclear staining alone. For inactive mTOR, P-mTOR and P-AKT, scoring was done for the cytoplasmic staining only (as the cases that showed combined cytoplasmic and nuclear staining were few and thus may be non-conclusive during statistical analysis) but for PTEN, nuclear staining along with cytoplasmic staining was scored. All cores were scored by two investigators (SS, AS) in a blinded setting. In a combined session, consensus on expression value was reached in all cases. Finally, the data was analyzed and correlated with the collected patient and tumor criteria. Neither direct patient communication nor any procedure was done on the patients.

***Statistical analysis:***

Analysis of data was done using SPSS program version 25. Quantitative data were presented as minimum, maximum, mean and SD (or Median and IQR for non-parametric data). Qualitative data were presented as count and percentage. Mann Whitney U test and Kruskal Wallis tests were used to compare quantitative variables between different groups. Chi square test (or Fisher Exact test) were used to compare qualitative data between different groups. P value less than or equal to 0.05 was considered statistically significant.

## Result

***Clinicopathologic characteristics***

Among the 166 patients, 60 patients were below the age of 70, and 104 were at or above 70 years with age range (49-92) and an average age of 72.63 years. The initial PSA level was more than 20 ng/L in 108 patients and less than 20 ng/L in 54 patients. Among the patients, 41 cases (29.7%) were identified with lymph node metastasis and 97 (70.3%) without lymph node metastasis. Bone metastases were seen in 73 cases (46.5 %). Gleason pattern 4 was the commonest and the highest in most cases presenting in (70 cases) 42.1% and (75 cases) 45.1% respectively. Total Gleason score 7 was the commonest and reported in 53 cases (31.9%).

The patients were divided into low, intermediate and high-risk groups based on initial serum PSA level whether <10 or more than 20 ng/ml, Gleason score / WHO grade group and tumor stage with most of the studied cases were in the high-risk group (126 cases / 75.9%).

The clinicopathological characteristics of the patients are presented in Table 2.

***The Expression of the different markers in the studies cases:***

***Regarding total mTOR:*** Most prostatic carcinoma cases showed positive stain (96.1%) whether diffuse cytoplasmic stain (n=135, 87.7%), (Fig. 1 A, B) or patchy stain (n=13, 8.4%) and only 6 cases, 3.9% were completely negative (Table 3). Occasional cases were showing nuclear stain, thus were ignored as were not significant for statistical analysis.

***Table 1: Antibodies and staining information for Ventana Benchmark® Ultra system automatic monostainer***

| Protein | Company | Cat. No. | Clone No | Clonality | Titer | antigen retrieval | Endogenous peroxidase activity blocking | primary incubation | Positive control |
|---|---|---|---|---|---|---|---|---|---|
| **Inactive mTOR** | abcam/Cambridge, UK | (ab218525) | 49F9 | Rabbit monoclonal | 1:200 | 32 min CC-1 at 95°C | 0.03%H2O2 /5min | 60 min at 36°C | Breast carcinoma |
| **p-mTOR (S2448)** | abcam/Cambridge, UK | ab109268 | D68F8 | Rabbit monoclonal | 1:1000 | 32 min CC-1 at 95°C | 0.03%H2O2 /5min | 32 min at 36°C | Breast carcinoma |
| **p-AKT (Ser473)** | abcam/Cambridge, UK | ab81283 | 236B4 | Rabbit monoclonal | 1:750 | 32 min CC-1 at 95°C | 0.03%H2O2 /5min | 60 min at 36°C | Breast carcinoma |
| **PTEN** | abcam/Cambridge, UK | ab32199 | Y184 | Rabbit monoclonal | 1:100 | 32 min CC-1 at 95°C | 0.03%H2O2 /5min | 60 min at 36°C | Proliferative endometrium |

There was no significant correlation between inactive mTOR and patient's criteria. There is a significant correlation was found between diffuse positive stain and Gleason pattern 4 as the commonest and the highest pattern as well as combined Gleason score 7 but this was suggested

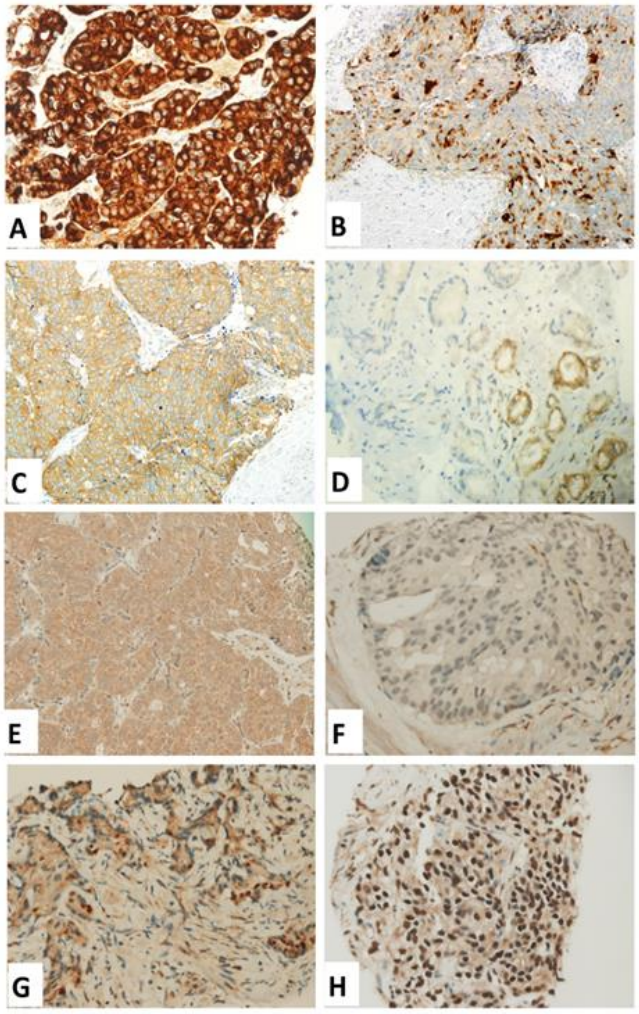


***Figure 1: A: Inactive mTOR showed diffuse positive cytoplasmic stain, x400. B: Inactive mTOR showed patchy positive cytoplasmic stain, x200. C: Phosphorylated mTOR showed diffuse cytoplasmic stain, x200. D: Phosphorylated mTOR showed patchy cytoplasmic stain, x2***

as pattern 4 and Gleason score 7 were commonly reported in many cases (Figure 2A), thus definite correlation can't be confirmed.

***Regarding p-mTOR:*** Most prostatic carcinoma cases showed positive stain (93.5%) whether diffuse cytoplasmic stain (n=112, 73.2%) or patchy staining (n= 31 cases, 20.3%) (Fig. 1C, D) with only 10 cases, 6.5% were negative (Table 3). There was mostly no significant correlation between the p-mTOR expression and the patient or tumor criteria except for Gleason pattern 4 (Figure 2B).

***Regarding p-AKT:*** Most cases showed positive cytoplasmic stain (n=139 cases, 95.9%) (Fig. 1E) while only 6 cases, 4.1% showed negative staining (Table 3). There was no significant correlation between p-AKT expression and the patient or tumor criteria (Figure 2C).

***Regarding PTEN:*** PTEN was diffusely expressed (retained stain) in 60 cases (44.8%) with PTEN loss whether patch in 38 cases (28.4%) or total loss in 36 cases (26.9%) (Fig. 1 F, G and H) (Table 3). No significant correlation was found between PTEN loss and the patient's or tumor criteria (Figure 2 D).

***Correlation of inactive mTOR, p-mTOR, p-AKT and PTEN expression with the clinicopathological parameters:***

The correlation between the immunohistochemical results for total mTOR, p-mTOR, p-AKT and PTEN with the patient and tumor criteria are shown in (Figure 2A, B, C and D).

***Correlation of the expression of different markers:***

The correlation of each marker with the rest of markers showed no significant correlation (Figure 3 A, B and C) except between phosphorylated mTOR and phosphorylated AKT expression with p value of 0.004. This is expected as phosphorylated AKT is the one activate / phosphorylate mTOR at serine 2448 residue.

## Discussion

In this study we investigated a cohort of 166 cases with prostatic adenocarcinoma for the activation of mTOR pathway using immunohistochemical technique for a panel of four antibodies (inactive mTOR, p-mTOR (Ser 2448), p-AKT (Ser 473) and PTEN) in correlation to clinicopathological parameters looking for a basis to use mTOR inhibitors as a therapeutic target for prostatic adenocarcinoma especially in castrate resistant prostatic cancer (CRPC) that showed no effective therapy till now. Kinkade et al [13] found that targeting AKT/mTOR and other singling markers in the mTOR pathway could suppress hormone-refractory prostate cancer in a preclinical mouse model. Shorning, et al [5] found that oncogenic activation of the phosphatidylinositol-3-kinase (PI3K), and mammalian target of rapamycin (mTOR) pathway is common in prostate cancer and implicated in tumor progression and therapeutic resistance. AKT is the main effector of PI3K. Thus, PI3K/AKT/mTOR-targeted therapies to prostate cancer is being explored. In this study, most prostate cancer cases showed either low or high positive expression for both inactive and p-mTOR with 93.5% and 96.1% respectively as well as positive expression for p-AKT in 95.9%. These results are supportive that this pathway is being activated during the prostate cancer development.

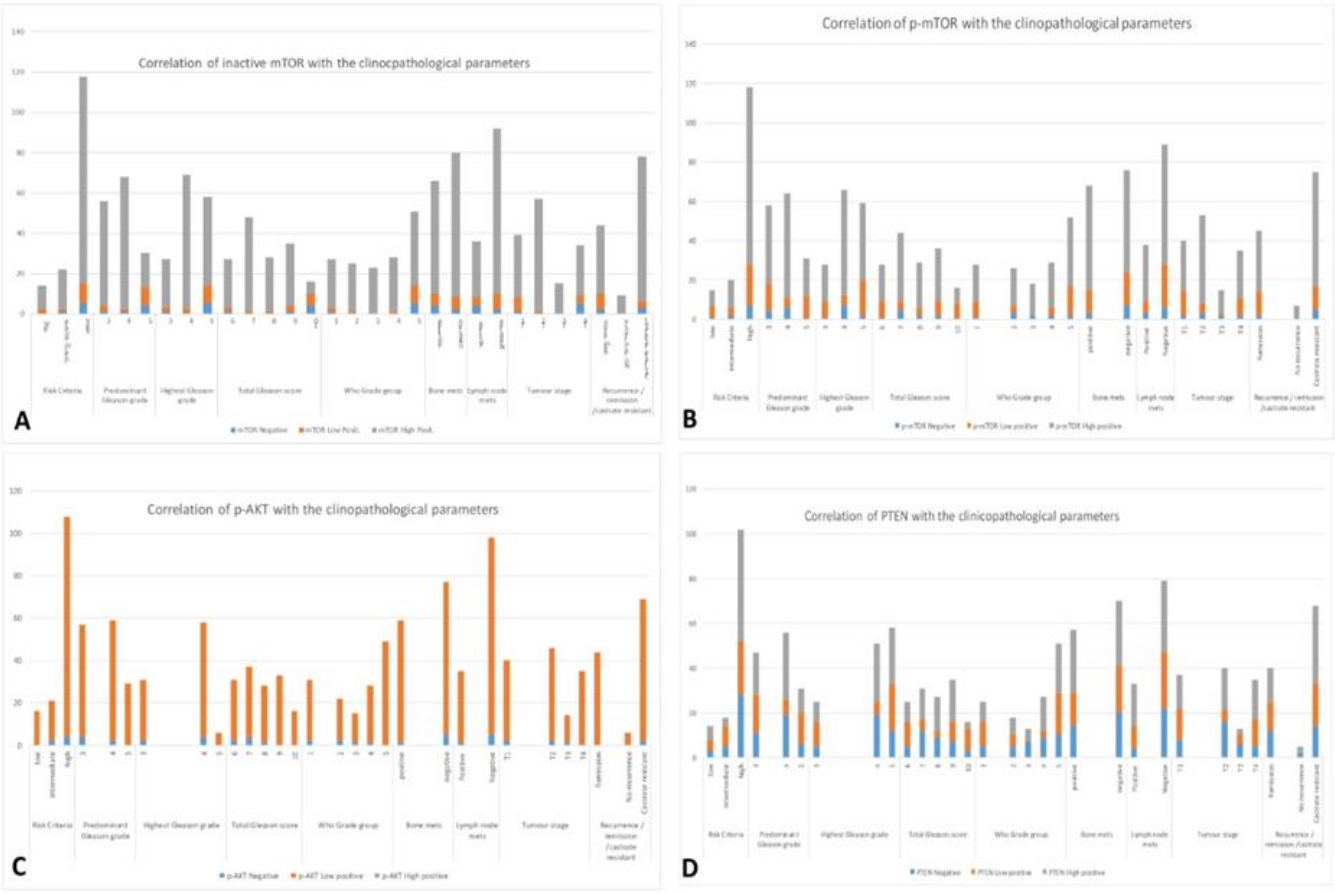


***Figure 2: Correlation of different markers with clinicopathological parameters: A) with inactive mTOR, B) with phosphor-mTOR, C) with phosphor-AKT and D) with PTEN loss.***

Dai et al [14] reported that in addition to the expression of at least one of mTOR pathway markers in prostate cancer patients, there is positive significant correlation between activation of mTOR pathway and the clinicopathological parameters. In fact, this is the most conflicting issue found in the literature. In this study, the activation of mTOR pathway through over expression of inactive mTOR, p-mTOR and p-AKT was mostly not significantly correlated with patient or tumor criteria, except with Gleason pattern 4 rather than 3 or 5. That was significantly correlated with diffuse expression of both inactive mTOR and p- mTOR. Whether this is significant for Gleason pattern 4 in similar way as cribriform growth of Gleason pattern 4 that considered unfavored prognostic parameter. It is a major predictive factor for distant metastasis and disease-specific death of prostate cancer in Gleason score 7 patients [15] or it is just accidental finding in view of widespread pattern 4 in the studies cases. Although, the latter is favored, further studies are also needed.

On the other hand, Stello et al [12] reported that activation of mTOR pathway in prostate cancer, is associated with favorable outcome. They found that high-risk and high stage patients have low mTOR activity.

In this study, we investigated the correlation of the mTOR pathway activation in relation to the different risk groups and we did not find any correlation. Also, we analyzed the correlation between the activated mTOR pathway (overexpression of inactive / phosphorylated m-TOR and phosphorylated AKT) and castrate resistant prostate cancer, but no significant correlation was found. This could be explained by positive expression of these markers in most cases (with 93.5%, 96.1% and 95.9% respectively) whether they were castrate resistant or not. Thus, we couldn't rule out benefit from the mTOR target therapy.

Armstrong, et al7 as well as Edlind and Hsieh [6] reported limited clinical efficacy of mTOR inhibitors in the castration resistant and neoadjuvant setting. Statz et al [16] published a systemic review article incorporating 14 different studies on mTOR inhibitors in castrate resistant prostate cancer, two of which combined with antiandrogen therapy. They reported minimal response to the treatment.

We suggest different explanations for this conflicting data regarding the correlation between the activation of mTOR

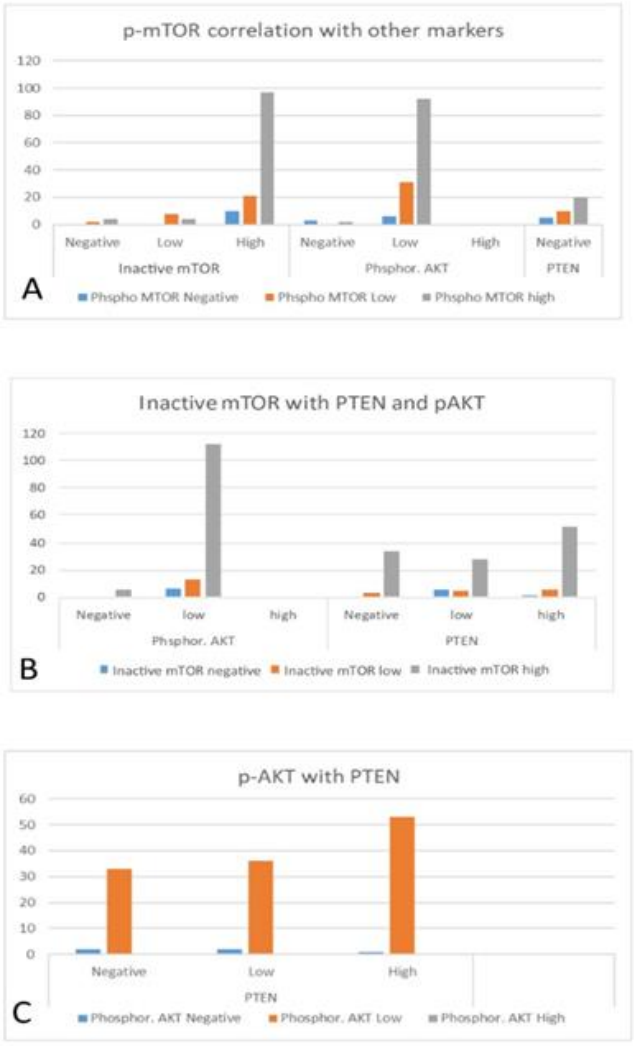


***Figure 3: Correlation of the expression of different markers; a) phospho-mTOR with other markers, b) inactive mTOR with PTEN and phospho-AKT and c) phospho-AKT and PTEN.***

pathway and favorable versus unfavorable patient outcome as well as suggested limited efficacy of mTOR inhibitor despite mTOR pathway activation. First cause, our study incorporated the main molecular markers of mTOR pathway, other molecular markers are being under discovery. Lu, et al [17] found tumor necrosis factor-α induced protein 8-like 2 (TIPE2) overexpression inhibiting the PI3K/AKT signaling pathway. Audet-Walsh, et al [18] found that an AR/mTOR complex promotes sterol regulatory element-binding transcription factor 1 (SREBF1) expression and function that could also be used as target therapy. Moreover, Manin, et al [19] explored the discovery of crosstalk between PTEN-sustained PI3K signaling and the AR pathways. Thus, there may be other molecular markers that control this pathway and determine patient outcome and response to therapy. Second issue is the possibility of the presence of nonfunctional protein, secondary to specific gene mutation. Thus, molecular / genetic study should be incorporated to rule out mutant nonfunctional proteins.

Another important issue, that our studied markers were expressed cytoplasmic with mostly negative nuclear stain in our cases. Audet-Walsh, et al [20] found that increased mTOR nuclear localization rather than cytoplasmic was detected in castration-resistant prostate cancer and metastases.

Our study showed that PTEN loss was detected in 55.3% of prostate cancer cases including primary and castrate resistance and this is in concordance with Vivanco and Sawyers [21] who found PTEN loss in 30% and 60% of primary and castration-resistant prostate cancer respectively. Although in their study, Vivano and Sawyers [21] showed association between PTEN loss and high Gleason score, our study showed no significant correlation between PTEN loss and high Gleason score. This can be attributed to the high prevalence of Gleason pattern 4 in our studied cases (42.2%).

Regarding the correlation between activation of mTOR pathway and PTEN loss, unfortunately, in view of activated mTOR pathway in most of our studies cases, no significant correlation was achieved. Jia et al [22] reported that patients with PTEN-deleted prostate cancer can benefit from drugs blocking PI3K signaling.

## Conclusion

This study demonstrated the activation of mTOR pathway in most prostate cancer patients, whatever their grade or stage and thus suggest early activation of this pathway with the possibility to the use mTOR inhibitors as line of treatment but with the unpredictable effect result. No significant correlation is found between activation of mTOR pathway or PTEN loss and tumor grade or stage whether high or low. This may add to the debate / conflicting data in literature concerning the favorable or unfavorable outcome of patients with activation of mTOR pathway. PTEN loss may be present in about half of prostate cancer cases. Although, mTOR pathway is inhibited by PTEN in interactive manner, this research may suggest different insults, with some lead to activation of mTOR pathway and others lead to PTEN loss.

***Table 2: Clinicopathologic Parameters of the patient:***

| Parameter | Criteria | Number | % | Total number |
|---|---|---|---|---|
| **Age** | Above 70 | 104 | 63% | 164 |
| | Below 70 | 60 | 37% | |
| **General condition** | Good / no organ failure | 43 | 53.75% | 80 |
| | Bad/ organ failure, medical condition, other cancer | 37 | 46.25% | |
| **Initial PSA level** | Above 20 | 108 | 66.66% | 162 |
| | Below 20 | 54 | 33.33% | |
| **Clinical Risk Groups** | High risk | 126 | 75.9% | 166 |
| | Intermediate risk | 23 | 13.85% | |
| | Low risk | 17 | 4.2% | |
| **Symptoms** | Obstructive | 43 | 55.8% | 77 |
| | Irritative | 4 | 5.2% | |
| | Both obstructive and irritative | 11 | 14.3% | |
| | High PSA | 2 | 2.6% | |
| | Follow up | 5 | 6.5% | |
| | Metastasis | 5 | 6.5% | |
| | Hematuria | 7 | 9.1% | |
| **Signs / PRE** | Hard nodular | 41 | 65.1% | 63 |
| | Benign feeling | 11 | 17.5% | |
| | Borderline /non-conclusive | 11 | 17.5% | |
| **Predominant Gleason pattern** | 3 | 63 | 37.9% | 166 |
| | 4 | 70 | 42.1% | |
| | 5 | 33 | 19.8% | |
| **Highest Gleason pattern** | 3 | 31 | 18.6% | 166 |
| | 4 | 75 | 45.1% | |
| | 5 | 60 | 36.1% | |
| **Total Gleason score** | 6 | 31 | 18.6% | 166 |
| | 7 | 53 | 31.9% | |
| | 8 | 29 | 17.4% | |
| | 9 | 37 | 22.2% | |
| | 10 | 16 | 9.6% | |
| **WHO Grade group** | 1 | 31 | 18.7% | 166 |
| | 2 | 29 | 17.5% | |
| | 3 | 23 | 13.9% | |
| | 4 | 30 | 18.1% | |
| | 5 | 53 | 31.9% | |
| **Percentage of tissue involved** | Mean 60 | Minimal 1% | Maximum 100% | 139 |
| **Bone metastasis** | Positive | 73 | 46.5% | 157 |
| | Negative | 84 | 53.5% | |
| **Lymph node metastasis** | Positive | 41 | 29.7% | 138 |
| | Negative | 97 | 70.3% | |
| **Tumour stage** | T1 | 42 | 26.9% | 156 |
| | T2 | 61 | 39.1% | |
| | T3 | 16 | 10.3% | |
| | T4 | 37 | 23.7% | |
| **Type of therapy** | Hormonal | 122 | 80.8% | 151 |
| | Chemotherapy | 21 | 13.8% | |
| | Radiation | 40 | 26.5% | |
| | TURP | 73 | 48.3% | |
| | Radical surgery | 18 | 11.9% | |
| **recurrence / remission / castrate resistant** | Remission | 51 | 36.6% | 140 |
| | Castrate resistant | 80 | 57% | |
| | No recurrence | 9 | 6.4% | |

***Table 3: the distribution of Immune histochemical results for tested markers***

| | IHC result | Number | % | Total |
|---|---|---|---|---|
| **Inactive mTOR** | Negative | 6 | 3.9% | 154 |
| | Positive patchy / low expression | 13 | 8.4% | |
| | Positive diffuse /high expression | 135 | 87.7% | |
| **p-mTOR** | Negative | 10 | 6.5% | 153 |
| | Positive patchy / low expression | 31 | 20.3% | |
| | Positive diffuse /high expression | 112 | 73.2% | |
| **p-AKT** | Negative | 6 | 4.1% | 148 |
| | Positive | 139 | 95.9% | |
| **PTEN** | Negative | 36 | 26.9% | 134 |
| | Positive patchy/ low expression | 38 | 28.4% | |
| | Positive diffuse / high expression | 60 | 44.8% | |

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**Corresponding Author:**

Dr. Somaia Ahmed Saad El Din (Associate Professor, MD, PhD)

Pathology Department, Faculty of Medicine, Ain Shams University, Abbassia Street, Cairo, Egypt

drsomaia2005@gmail.com

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