RESEARCH ARTICLE

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Evaluation of the Expression *EGFR, HER2/NEU* and the End Effector *ERK* of the RAS/RAF/MAP Kinase Pathway in Prostatic Adenocarcinoma for a Possible Role as New Target Therapy

Asem Shalaby[1,2]*, Heba A Mahmoud[3], Shaymaa G Sayed[2], Khalid Al Hashmi[4], Shadia Al Sinawi[2], Suaad Al Badi[2], Afrah Al Rashdi[2], Samya Al Husaini[2], Hajer Al Badi[2], Somaia Ahmed Saad El-Din[3,5]

Abstract

The alterations of EGFR and *HER2/neu* as growth factor receptors and the cytoplasmic signal transduction proteins of RAS/RAF/MAP kinases including its end effector molecule (*ERK*) are important in the carcinogenesis of many tumors. The activation of these protooncogenes in prostate cancer is still under investigation. The aim of this work was to study *EGFR*, HER2- neu, inactive (non-phosphorylated) and active (phosphorylated) *ERK* expression in prostatic adenocarcinomas in correlation to the clinical and pathological parameters. **Methods:** Immunohistochemistry- using tissue microarrays- for *EGFR*, *HER2/neu*, non-phosphorylated, and phosphor-*ERK*, was performed on tissues from 166 patients- with primary prostatic adenocarcinoma with no prior treatment-. The results of different markers expression were correlated with the clinical and pathological parameters and were analyzed statistically. **Results:** The prostatic tissue showed *EGFR*, HER2 neu, phosphorylated and non-phosphorylated *ERK* expression in 8.4%, 1.4%, 78.2%, and 83.4% respectively whether low (patchy) or high expression (diffuse). There were no significant correlations found between patient characteristics and expression of the tested markers. The negative immune reactivity for non-phosphorylated *ERK* and *EGFR*- was significantly correlated with high tumor stage (p values 0.03 and 0.01, respectively). **Conclusion:** *EGFR* and *HER2/neu* may play a limited role in prostatic adenocarcinoma as they showed positive expression in a limited number of the examined tissues specifically HER2neu. The expression of non-phosphorylated *ERK* (mostly weak to moderate) and phosphorylated *ERK* (mostly moderate to strong)- was appreciated in most cases. Thus, we suggest that anti-*EGFR* drugs may have a limited role in the treatment of castrate-resistant prostate cancer, but anti-MEK/*ERK* drugs may have more promising role as a target therapy. It is recommended to perform further molecular testing to elucidate the exact mechanism and significance of these markers.

Keywords: Prostate tumorigenesis- *EGFR*, HER2 neu- *ERK*- therapeutic target- castrate resistant prostate cancer

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Introduction

Prostate cancer is the second leading cause of cancer-related death in the United States with decreasing incidence of prostate cancer overall since 2000 but an increasing incidence of wide metastatic disease [1]. Metastatic castration-resistant prostate cancer (m CRPC) is incurable with no effective therapy till now beyond hormonal treatment. Thus, there is an urgent need to develop new effective therapy [2].

The genetic changes in *EGFR* gene- either mutations or amplification- is implicated in the progression of many types of cancers and currently the *EGFR* inhibitors such as gefitinib and cetuximab are in clinical use for metastatic colorectal cancer and non-small cell lung cancer [3].

Moreover, it is also reported that in prostatic carcinoma, *EGFR* increased expression correlates with the higher Gleason scores and the advanced stages of the disease [4] and that *EGFR* activation is associated with metastatic progression and recurrence [5].

The literature revealed a controversy regarding the response of castration-resistant prostate cancer (CRPC) to *EGFR* inhibitors; while in phase 2 clinical trial, gefitinib showed no response as reflected by PSA level or other

*[1] Department of Pathology, Faculty of Medicine, Mansoura University, Mansoura, Egypt. [2]Department of Pathology, College of Medicine and Health Sciences, Sultan Qaboos University, Muscat, Oman. [3]Department of Pathology, Faculty of Medicine, Ain Shams University, Cairo, Egypt. [4] Department of Hematology and Medical Oncology, Armed Forces Hospital, Muscat, Oman. [5]Department of Histopathology, Armed Forces Hospital, Muscat, Oman. *For Correspondence: asemali444@yahoo.com*

objective disease measures in patients with CRPC [6]. In a second trial, cetuximab showed a well detected decline in PSA levels in many cases and improved patient free survival in patients with overexpressed of *EGFR* [7].

Mutations in downstream molecules in the signaling pathway have showed good correlation with the lack of response to cetuximab [8]. The Ras/Raf/ MEK/ *ERK* pathway represents the most important signaling mechanism among all mitogen-activated protein kinase (MAPK) transmission pathways which play a role in signaling cascades and transmit extracellular signals to intracellular targets. Thus, it plays a crucial role in normal cell survival and the development of tumors [9,10].

The MAPK pathways generally include three main kinases, *ERK1/2* is one of them with the phosphorylation of *ERK* can be used as a common endpoint measurement for the activation of this pathway. The extracellular signal-regulated kinases *ERK*1 and *ERK*2 are ubiquitous serine-threonine kinases that regulate cellular signaling in both normal and pathologic conditions [9,11]. Since ERK1 and *ERK2* are very similar, the *ERK* singular form is used in the current study, although the two subtypes do exist.

Increased activation of the *RAS/RAF/MEK/ERK* pathway has shown an association with poor prognosis and androgen independence in prostate cancer [3] Increased expression members of MAPK pathway and high levels of phosphorylated *ERK1/ ERK 2* were observed in m CRPCs [3,12]. So, therapeutic targeting of the MEK/ERK pathway could be a viable strategy for those with m CRPC, and trametinib (the MEK inhibitor) is currently being tested in a phase two trial for patients with m CRPC [3].

This study assesses the immunohistochemical expression of *EGFR*, *HER2*, non-phosphorylated and phosphorylated ERK in prostate acinar adenocarcinoma along with the different patients' clinicopathological parameters- in a trial to use the new target therapy of *EGFR* and/or MEK inhibitors in the management of advanced stage patients especially those with castrate resistant prostate cancer whom failure of other treatment modalities is encountered.

Materials and Methods

Patient samples

The study was approved by the Ethical Committees at Armed Forces Hospital (AFH), Muscat, Oman with reference number AFMS-MREC006/2020. This study included 166 prostatic acinar adenocarcinoma cases that were collected from Pathology archives starting from the beginning of 2007 till the end of 2018.

Inclusion criteria

Patients with available clinical information along with available tissue material in the blocks were included in this study. The clinical information that was extracted including; patient age, signs and symptoms, PAS level before and after treatment. The histopathological parameters were assessed including; the Gleason grade, combined Gleason score, tumor percentage, tumor stage and WHO grade group. Then, the clinical risk group was determined. As the general patient condition can affect the patient therapeutic plan, any patient with organ failure was also mentioned. As this research is targeting the castrate resistant patients, therapy that was taken was extracted in brief including its type that may be hormonal, radiological, or surgical or if any chemotherapy had been taken along with patient response by follow up PSA level to determined patient with remission and relapse or recurrence after curative surgery.

Exclusion criteria

Any patients with unavailable or minimal tissue material in the paraffin blocks- were excluded from this study.

Tissue microarray (TMA) construction

Revision of hematoxylin and eosin-stained slides of the 166 prostatic acinar adenocarcinoma cases that were extracted from the Pathology archives at Sultan Qaboos University and Armed Forces Hospital, Muscat, Oman- was done. Selection of the blocks that showed available tissue material was done. Mostly, 2 blocks for each patient were retracted (thus if the tissue material of one core was lost during preparation or was not enough, there would be another core). Control specimens were used as negative marker control including 20 cases of non-neoplastic prostatic tissue. As well as 5 cases of invasive duct carcinoma which were Her2-neu positive (score 3) and they were positive for both inactive (non-phosphorylated) and active (phosphorylated) ERK (positive control for these markers). Also, 3 cases of proliferative endometrium (as positive control for *EGFR*) were included. Many other non-neoplastic tissues were used for tissue microarray mapping including 3 cases of tonsillar tissue, 3 cases of colonic mucosa, 3 cases of kidney, 3 cases of skin, 3 cases of testis and 3 cases of thyroid tissue.

Then, preparation of the recipient tissue microarray paraffin block is done by cutting two or three cylindrical cores, each about 1 mm from the selected area at the donor paraffin blocks (area of non-necrotizing invasive malignancy with good cellularity- were selected by examining the corresponding slides) using Manual Tissue Arrayer MTA-1 from Estigen OU, Tiigi 61b, 50410 Tartu, Estonia. Finally, we had prepared six TMA paraffin blocks, each one showed 90 malignant and control cylindrical tissue cores arranged in specific way (according to the prepared map) to be able to code the malignant cores.

Immunohistochemistry and interpretation

Tissue sections (5 μm) were mounted on amino-acetyl silane-coated glass slides (Starfrost, Berlin, Germany), Sections are kept in hot oven over the night along with xylene for dewaxing, then descending grades of alcohol and distilled water for rehydration. Then, application of the primary antibodies for each of the studied markers (*EGFR*, *HER2-neu*, non-phosphorylated and phosphorylated *ERK*)- was done guided by the provided Ventana Benchmark protocol using Utra system automated monostainer (Ventana Medical Systems) (Table 1). Examination of the positive and negative control samples- was done initially followed by examination of neoplastic cores in corresponding to the designated map.

Both, intensity of the stain (mild, moderate, or strong) and the extent of the positive neoplastic cells- were evaluated by two different investigators, if interobserver variation encountered, one more investigator was included. As each case was presented by two or three cores, if different stain was found among the examined cores for the same case, an average was considered. Regarding *EGFR* interpretation: Negative result was considered if less than 1% of the cells were positive. Then categorization of the cases was done into 3 groups: Low expression (1-10%), Moderate expression (10-50), High expression (more than 50%) [13].

In this study, the low (1-10%) and moderate expression (10-50%) groups- were categorized as patchy / low expression group along with the high expression group (more than 50%) for statistical proposal. Any membranous and / or cytoplasmic stain was interpreted as positive [14,15].

Regarding *HER2*/NEU, only complete strong membranous stain in more than 10% of the cells- was considered positive as in breast carcinoma cases [16].

Regarding *ERK* (both non-phosphorylated and phosphorylated), The negative stain was considered if no staining or less than 10% stained cells are encountered. Positive stain was considered if more than 10% of neoplastic cells were positive. Then positive results were further divided into low expression / patchy if less than 60% of the neoplastic cells were positive and high expression (diffuse) if more than 60% of the cells were positive [17].

The final interpreted immunohistochemical markers results were analyzed along with the collected patient data and the tumor histopathological features to find out any signification correlation. The patient consent was not applicable as there was no direct patient communication.

Statistical analysis

There were 166 retrospective cases underwent cross section study. SPSS program version 25 was used for study analysis. The mean, maximum and minimum and SD (or Median and IQR for non-parametric data) were used for the quantitative data. While counts and percentage were considered for the qualitative data. For comparing the quantitative variables between different groups, the Mann Whitney U test and Kruskal Wallis tests- were used. While, Chi-square test (or Fisher Exact test) was used to compare qualitative data between different groups. Statistically significant P value was considered if less than or equal to 0.05.

Results

Clinicopathologic characteristics

In the present study, regarding the patient clinical characteristic features, most of the studied cases were older than 70 years (63%), with high serum PSA level exceeding 20 ng/ml (66.6%) and with bone metastatic disease (46.5%). Thus, most cases were considered clinically in the high-risk group (75.9%) (depending on the high serum PSA, high Gleason score and high WHO grade group as well as high tumor stage).

Regarding the histopathological features of the cases, the commonest and the highest Gleason pattens were 4 representing (42.1 and 45.1 %) respectively along with combined Gleason Score 7 presenting (31.9%). Table 2 showed all patients clinical and histopathological features.

Markers expression in the studied cases and their correlation with the patients and tumor criteria
Regarding EGFR

Most prostatic carcinoma cases showed negative staining (91.5%). The positive cases mostly showed low expression representing about 4.9% with less evident cases showing high expression about 3.5%. (Table 3, Figure 1). No significant correlation was found between *EGFR* expression with any of the patient or tumor criteria. On the other hand, a significant correlation was found between negative *EGFR* expression and high tumor stage (Figure 2).

Regarding Her2-neu

Almost all prostatic carcinoma cases showed negative staining (98.6%) and only 2 cases representing (1.4%) showed positive staining. Thus, a statistical correlation between Her2-neu expression and the patient's or tumor criteria could not be done (Figure 1).

Regarding phosphorylated ERK

The phosphorylated ERK1/ERK2 positive immunoreactivity- was cytoplasmic and nuclear in most cases with few cases showing only nuclear stain or only cytoplasmic stain. While non-phosphorylated ERK immune reactivity was only cytoplasmic. Also, the expression of phosphorylated *ERK* was mostly moderate to strong stain, while, the expression of non-phosphorylated *ERK* was mostly weak to moderate stain. Thus, in the present study, we don't segregate cases by stain site whether cytoplasmic or nuclear or intensity. We categorized the cases into either low expression (patchy) if the positive reactivity affects less than 60% of the neoplastic cells and high expression (diffuse) if the positive reactivity affects 60% or more of the neoplastic cells.

Most of the cases showed positive cytoplasmic and nuclear staining, either patchy (48.5%) or diffuse (29.7%) (Table 3, Figure 1).There was no significant correlation between phosphorylated *ERK* expression and the patient criteria. While the only significant correlation found as regards tumor criteria was between the negative expression of phosphorylated *ERK* and the high stage of the tumor (Figure 2).

Regarding non-phosphorylated ERK

Most of the cases showed positive cytoplasmic staining, either patchy (61.5%) or diffuse (21.9%) (Table 3, Figure 1). No significant correlation was found between non-phosphorylated *ERK* expression and the patient's or tumor criteria (Figure 2).

Correlation of the expression of different markers

The correlation of each marker with the rest of the markers showed no significant correlation (Figure3).

Table 1. The Staining Protocol and Antibodies information for Ventana Benchmark® Ultra system Automatic Stainer

Protein	Manufacturer	Catalogue number	Clone	Clonality	dilution	antigen retrieval method	Blocking of peroxidase activity	incubation	Positive control
EGFR	abcam/ Cambridge, UK	ab30	EGFR1	mouse monoclonal	1:100	30 min CC-1 at 95°C	0.03%H2O2 /5min	60 min at 35°C	Proliferative endometrium
HER2neu	abcam/ Cambridge, UK	ab8054	CB11]	Rabbit monoclonal	1: 50	30 min CC-1 at 95°C	0.03%H2O2 /5min	60 min at 36°C	Breast carcinoma
Phosphorylated ERK (ERK1 + ERK2)	abcam/ Cambridge, UK	ab54230	ERK-7D8	mouse monoclonal	1:50	30 min CC-1 at 95°C	0.03%H2O2-methanole /15min	60 min at 35°C	Breast carcinoma
non-phosphorylated ERK (ERK1 + ERK2)	abcam/ Cambridge, UK	ab32538	E337	Rabbit monoclonal	1:50	30 min CC-1 at 95°C	0.03%H2O2 /5min	60 min at 35°C	Breast carcinoma

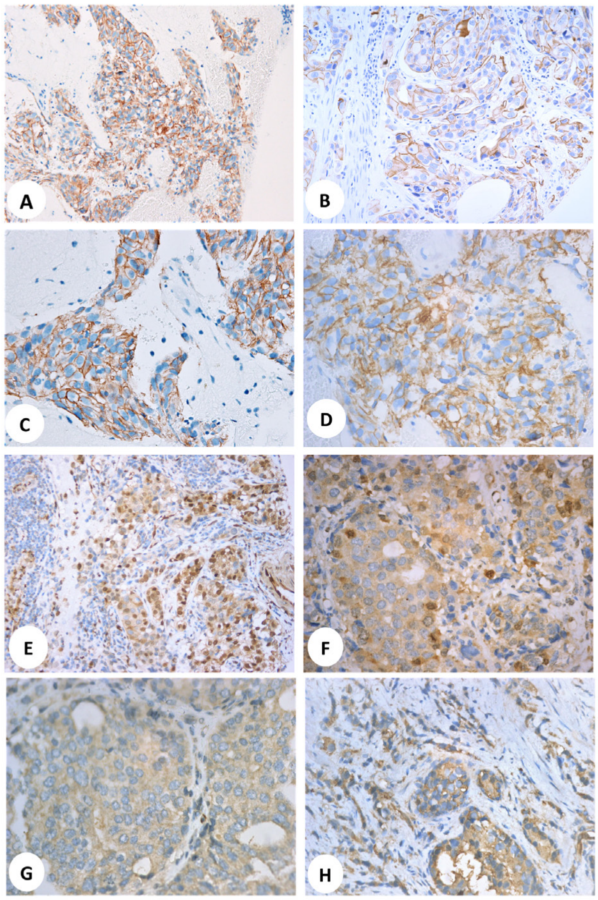

Figure1. Photomicrographs of Various Immune Histochemical Markers, (A) EGFR diffuse weak to moderate cytoplasmic and membranous stain, x400, (B) EGFR patchy positive membranous and cytoplasmic stain, x400, (C) HER2/neu moderate to strong membranous stain in more than 10% of the neoplastic cells, x400, (D) HER2/neu strong membranous stain in more than 10% of the neoplastic cells, x400, (E) phosphorylated ERK diffuse positive nuclear and cytoplasmic stain, x400, (F) phosphorylated ERK patchy positive nuclear and cytoplasmic stain, x400, (G) non-phosphorylated ERK showed diffuse positive cytoplasmic only stain, x400 and (H) non-phosphorylated ERK patchy cytoplasmic stain, x400.

Discussion

This study incorporated 166 cases of prostatic adenocarcinoma for whom an immunohistochemical study were done for *EGFR*, *HER2-neu*, and the end effector *ERK* (both non-phosphorylated and the phosphorylated) of the cell signaling Ras/Raf/MEK/*ERK* pathway. All clinical and histopathological features were also analyzed with especial concentration on the cases showed castrate resistant prostate cancer or recurrence especially if associated with organ failure, for whom a new safer therapy is needed. This study was searching for the expression level of *EGFR*, *HER2-neu* and *ERK* for a possible role of *EGFR* inhibitors and/or MEK inhibitors as a therapeutic target for castrate resistant prostatic adenocarcinoma.

This study showed that most prostatic adenocarcinoma neither expresses *EGFR* nor *HER2-neu* (only 8.4% and

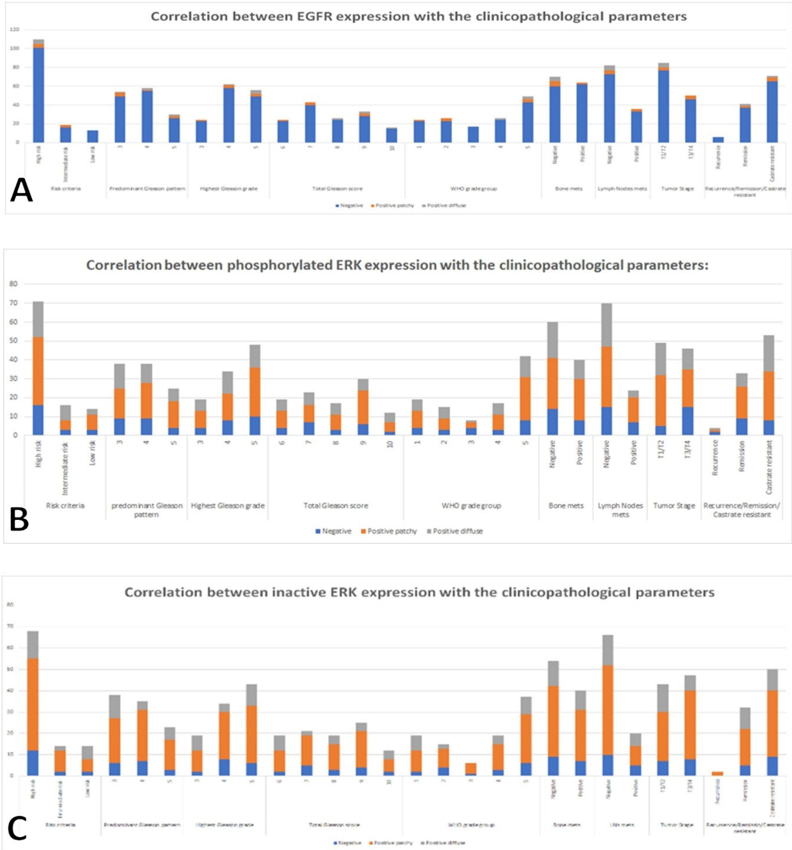

Figure 2. The Correlation between the Different Studied Immunohistochemical Markers with the Clinical and Pathological Features of the Studied cases (A) with EGFR, (B) phosphorylated-ERK and (C) with non-phosphorylated ERK

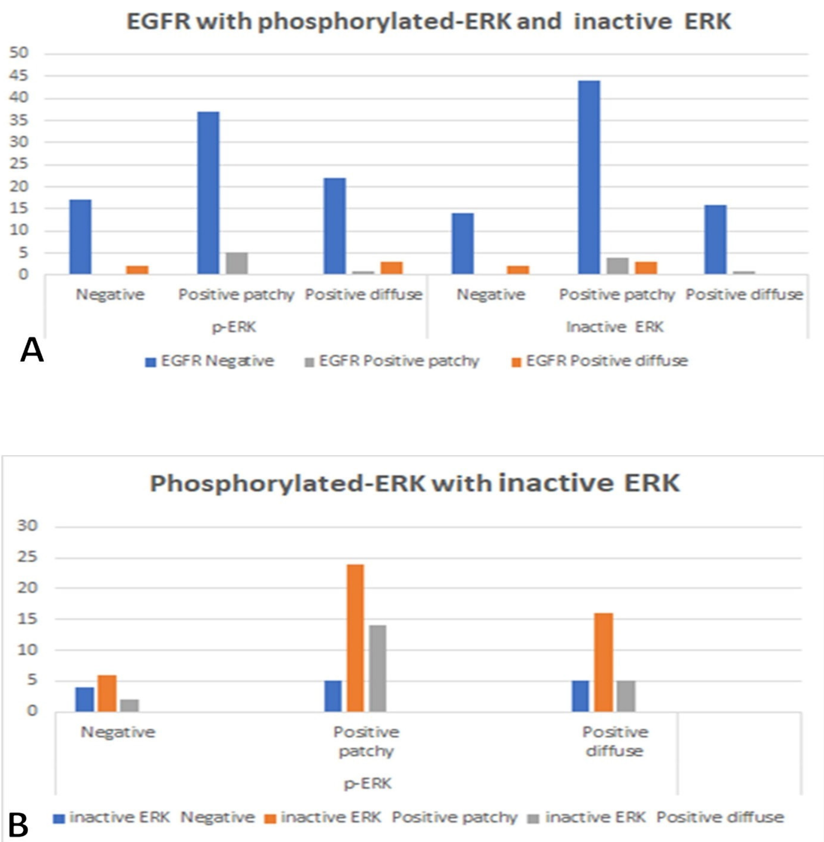

Figure 3. Correlation between the Different Markers Expression; (A) EGFR with non-phosphorylated ERK and phosphorylated-ERK and (B) non-phosphorylated ERK with phosphorylated-ERK.

Table 2. The Summary of Clinical and Histopathological Features of the Studied Samples

	Criteria	Number	Percentage	Total
Age	< 70	60	37%	164
	> 70	104	63%	
General status	Bad medical condition, organ failure, other cancer	37	46.25%	80
	Good / no organ failure	43	53.75%	
Initial serum PSA	< 20	54	33.33%	162
	> 20	108	66.66%	
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
	Both obstructive and irritative	11	14.3%	
	Hematuria	7	9.1%	
	Follow up	5	6.5%	
	Metastasis	5	6.5%	
	Irritative	4	5.2%	
	High PSA level	2	2.6%	
PRE / per rectal examination	Hard nodular	41	65.1%	63
	Benign feeling	11	17.5%	
	Borderline /non-conclusive	11	17.5%	
Commonest Gleason pattern	$3.00	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%	
% of involved tissue	Mean (60)	Minimum1%	Maximum100%	139
Bony metastasis	Negative	84	53.5%	157
	Positive	73	46.5%	
Lymph node metastasis	Negative	97	70.3%	138
	Positive	41	29.7%	
Tumor stage	T1	42	26.9%	156
	T2	61	39.1%	
	T3	16	10.3%	
	T4	37	23.7%	
Type of therapy	Hormones	122	80.8%	151
	TURP	73	48.3%	
	Radiation	40	26.5%	
	Chemotherapy	21	13.8%	
	Radical surgery	18	11.9%	
Castrate resistant / Remission / Recurrence	Castrate resistant	80	57%	140
	Remission	51	36.6%	
	No recurrence	9	6.4%	

Table 3. The Immunohistochemical Results of the Examined Markers

	IHC result	Number	%	Total
EGFR	Negative	130	91.5%	142
	Positive patchy	7	4.9%	
	Positive diffuse	5	3.5%	
HER2	Negative	145	98.6%	147
	Positive	2	1.4 %	
phosphorylated ERK	Negative	22	21.8%	101
	Positive patchy	49	48.5%	
	Positive diffuse	30	29.7%	
non-phosphorylated ERK	Negative	16	16.7%	96
	Positive patchy	59	61.5%	
	Positive diffuse	21	21.9%	

1.4% were positive respectively). In concordance with our result, Back et al, 18 reported no amplification of the *EGFR* or HER2 genes in their studied prostate cancer specimens. On the other hand, Di Lorenzo et al. [4] found *EGFR* expression in 41.4% of non-metastatic prostatic carcinoma treated with radical prostatectomy and 75.9% of those who were treated by hormonal therapy followed by radical prostatectomy [14].

Di Lorenzo et al. [4] have found significant associations between *EGFR* overexpression and poor prognostic indicators as higher Gleason score, perineural invasion, more tissue involvement by carcinoma, and disease recurrence, thus proving a strong prognostic significance of expression of *EGFR* in prostatic cancer. On contrary, this result didn't find any significant correlation between the positive expression of *EGFR* and patient criteria or the unfavorable prognostic features of the tumor. This could be due to the few numbers of positive cases (8.4%). In concordance with our results, Back et al. [18] found no significant association of *EGFR* expression with other clinicopathologic parameters except its inverse correlation with androgen receptor expression.

Although the results of the current study do not support that *EGFR* or *HER2-neu* are driving molecular changes in prostatic cancer, yet the role of *EGFR* as a prognostic biomarker can't be ignored as Cathomas et al. [7] found that targeting *EGFR* resulted in a well-detected PSA decline in many cases, and improved PFS in patients with overexpression of *EGFR*. Further molecular studies are recommended to assess any significant pathological *EGFR* variant thus, patients can get benefit from *EGFR* tyrosine kinase inhibitors.

In the present study, most prostate cancer cases showed either patchy or diffuse positive expression for both phosphorylated and non-phosphorylated *ERK* with 78.2% and 83.4% respectively, In Raf/MEK/*ERK* signaling pathway, *ERK*1 represents the immediate downstream target of druggable MEK1/2 which is druggable with trametinib (an approved therapeutic agent for melanoma) [18]. In concordance with our result, Nickols et al. [2] have found that patients with castration-resistant prostate cancer have higher levels of phosphorylated *ERK*1/2 compared to patients with untreated primary prostate cancer. Thus, therapeutic targeting of the Raf/MEK/*ERK* pathway could be a valuable treatment for patients with castration-resistant metastatic prostate cancer. This hypothesis is under ongoing phase II trial tests [18]. Moreover, up-regulation of *ERK*1/2 signaling has been shown to be one such mediator in resistant clones of previously cetuximab-sensitive cell lines (Anti *EGFR* tyrosine inhibitor) [19].

The main limiting factor of this study was that lack of performing molecular studies to investigate the association of positive IHC expression with molecular abnormalities and gene amplifications. Identification of the underlying gene amplification may also help in the proper selection of patients that can benefit from anti-*EGFR* or anti-MEK therapies.

Another important issue that limits this research is that the studied markers were expressed cytoplasmic with or without nuclear staining and segregation of nuclear stained cases wasn't done. Smith et al. [20] found that metastatic and castrate resistant prostate cancer showed increased nuclear localization rather than cytoplasmic localization of P-*ERK*. They suggested that p-*ERK* enters the nucleus in cancer cells to promote proliferation, while staying in the cytoplasm of the normal cells with no significant function effect as normal cells express lower levels of nuclear pore complex proteins and the nuclear transport factors, Thus P- *ERK* dissociated from nuclear entry, which is a rate-limiting step and thus cytoplasmic p-*ERK* positivity may not suggest functional activity of P-*ERK* and may not correlate with bad prognosis. Tanaka et al. [21] In the present study, we didn't segregate our cases with only nuclear staining as most cases were showing both nuclear and cytoplasmic stain. Extended larger studies correlating the IHC expression with the molecular study are needed concerning only nuclear P-*ERK* stain.

In conclusion, this study demonstrated that the immunohistochemical expression of *EGFR* and HER2neu are low in prostatic adenocarcinoma even in the castrate-resistant cases. But even though, as castrate-resistant patients had no more optional therapy, the study of *EGFR* on both IHC and molecular levels may suggest benefit from the new *EGFR* tyrosine kinase target therapy. On the other hand, non-phosphorylated *ERK* and phosphorylated *ERK* were appreciated in most studies' cases. This may suggest a promising role of MEK inhibitors. Taking into consideration the need for extended studies that concern only the nuclear *ERK* stain and correlate the IHC expression with molecular tests.

Author Contribution Statement

Conceptualization: Shalaby A, Saad El-Din SA. Data curation: Shalaby A, Saad El-Din SA, Al Hashmi K. Formal analysis: Shalaby A, Saad El-Din SA. Investigation: Al Sinawi S, Sayed S, Al Badi S. Methodology: Afrah Al Rashdi A, Al Husaini S, Albadi H. Project administration: Shalaby A. Resources: Shalaby A, Saad El-Din SA. Supervision: Shalaby A, Saad El-Din SS. Writing - original draft: Saad El-Din SA, Mahmoud HA. Writing - review & editing: All authors.

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Approval

The study was approved by the Ethical Committees at Armed Forces Hospital (AFH), Muscat, Oman with reference number AFMS-MREC006/2020.

Availability of the data

All data are available upon request.

Conflict of interest

The authors disclose no conflict of interest.

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