RESEARCH ARTICLE

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Immunohistochemical Expression of Alpha-Methyl-Coa (AMACR) and ERG in Prostatic Adenocarcinoma and Prostatic Hyperplasia: A Comparative Study

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Abstract

Objective: This observational comparative study aimed at investigating the diagnostic accuracy of ERG in differentiating benign and malignant prostatic lesions and comparing it to the diagnostic accuracy of AMACR. We also aimed at comparing AMACR and ERG expression to Gleason grade of the carcinoma cases. **Methods:** Seventy- two cases (22 prostatic hyperplasia and 50 prostatic carcinoma) were collected from the pathology department at Cairo university. The cases were immunostained by antibodies against AMACR and ERG. Immunohistochemical expressions of both markers were differentially examined in benign and malignant cases, compared to each other's, as well as, to the grade group of the malignant cases. **Results:** AMACR showed 62% sensitivity and 86.4% specificity for the diagnosis of PC, with a statistically significant differential expression in benign and malignant lesions (P=0.001). Its expression also correlated significantly with the age (p=0.007), Gleason grade (P=0.006) and perineural invasion (P=0.011). Although ERG showed 100% specificity to PC with no expression in hyperplasia cases, it showed only 22% sensitivity for PC cases. ERG expression also showed statistically significant correlation with the Gleason grade. No association between ERG and AMACR expression was detected in our study (P=0.151). Regarding the diagnostic accuracy, although ERG accuracy was much lower than that of AMACR, combining both markers yielded a higher diagnostic accuracy. **Conclusion:** Although ERG proved no superior value than AMACR in diagnosing prostatic lesions, combining both markers may lead to higher diagnostic accuracy owing to higher ERG specificity for PC.

Keywords: Histopathological diagnosis - prostatic lesions - AMACR - ERG - immunohistochemistry

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Introduction

Prostate carcinoma (PC) is the most commonly diagnosed cancer in men worldwide (Ferlay et al., 2015). Based on GLOBOCAN 2018 estimates, 1,276,106 new cases of PC were reported worldwide in 2018, with higher prevalence in the developed countries. It is the fifth most common cause of cancer related death globally (Rawla, 2019). In Egypt, PC constituted between 4.27% -5.25% of male malignancies ranking as the third most common male malignancy based on data of the National Cancer Registry Program of the Egyptian population (Ibrahim et al., 2019).

Prostatic carcinoma is a heterogeneous disease with a variable spectrum of histologic as well as biological features (Abdel-Hady et al., 2017). Benign mimics of prostate carcinoma may include normal structures such as seminal vesicles, inflammatory processes, glandular hyperplasia, atrophy and metaplasia. The most challenging of these mimics are the small glandular proliferations, such as atypical adenomatous hyperplasia, atrophy, partial atrophy, post-atrophic hyperplasia and basal cell hyperplasia (Trpkov, 2018).

In some cases, especially when the lesion is minimally represented, the differentiation between benign and malignant glands may be challenging (Gouda and Eloseily, 2019), as the diagnosis requires a combination of multiple features such as growth pattern, prominence of nucleoli and the presence or absence of basal cells. The accuracy of pathologic diagnosis of PC may be improved by the application of a more reliable tumor-specific immunohistochemical marker (Rathod et al., 2019).

One of the identified immunohistochemical markers of PC is the overexpression of alpha-methyl acyl CoA racemase (AMACR). AMACR catalyzes the conversion of (2R)-methylacyl-CoA to the 2S isomer for its degradation through the β-oxidation pathway. For unknown reasons, overexpression of AMACR occurs in 90 % of PC and high-grade prostatic intraepithelial neoplasia. Since this discovery, AMACR immunohistochemical staining,

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usually combined with a basal cell marker, has become a standard practice in confirming the diagnosis of PC in cases of ambiguous morphology (Box et al., 2016).

Among the limitations of using basal cell markers in differentiating benign and malignant prostatic lesions is the discontinuous or patchy presence of basal cells in the benign glands. So, negative basal cell markers in glands that are suspicious of cancer is not proof of their malignancy as benign glands may not show uniform basal cell markers expression (Hasan et al., 2020).

Regarding AMACR, although its sensitivity in staining PC is reported to range from 82% to 100% (Samarska and Epstein, 2023), its expression in PC is heterogenous. Additionally, hyperplastic glands and more importantly typical PC mimics such as partial atrophy and adenosis may display weak to moderate AMACR expression (Kristiansen, 2018). Thus, the correct interpretation of these markers is highly dependent on the morphological context to avoid false positive or negative results. This makes the addition of other diagnostic markers useful for proper stratification of benign and malignant prostatic lesions.

The E-26 transformation-specific (ETS) family member ERG transcription factor has physiological roles during development, as well as, in the vascular and hematopoietic systems. ERG oncogenic activity is documented in several malignancies, including Ewing's sarcoma, leukemia and prostatic carcinoma (Lorenzen et al., 2022).

In PC, ERG rearrangements with androgen-regulated genes mostly transmembrane serine protease 2 (TMPRSS2) characterize a subset of cases across disease progression and result in androgen receptor (AR)-mediated ERG overexpression in carcinoma cells. Importantly, PC cells overexpressing ERG are dependent on ERG activity for survival, further highlighting its therapeutic potential (Lorenzen et al., 2022).

Studies assessing the expression of ERG by immunohistochemistry and fluorescent In situ Hybridization (FISH) concluded ERG detection by immunohistochemistry in prostate cancer is highly predictive of ERG rearrangement as assessed by FISH (Falzarano et al., 2011).

This study aimed at investigating the immunohistochemical expression AMACR and ERG in prostatic acinar adenocarcinoma and prostatic hyperplasia to compare the diagnostic validity of both markers in differentiating benign and malignant prostatic lesions and also to detect the diagnostic yield of combining both markers. We also aimed to correlate the expression of both markers with the grade group of carcinoma cases to detect their prognostic significance and the possible benefit of using ERG targeted therapy in PC cases.

Materials and Methods

Cases Collection

This study is an observational analytical case control one. Approval from research ethics committee (REC) at faculty of medicine, Cairo university (REC code: N-80-2022) was obtained before starting. 72 cases (22 prostatic hyperplasia and 50 prostatic carcinoma) were included in this study.

Exclusion criteria

Biopsies with extensive necrosis, crushing or cautery artifact.

Histopathologic Examination

Formalin fixed paraffin embedded blocks were prepared from the collected cases. To preserve the patients' privacy, the names of the patients were replaced by an ID number. Only this number was used afterwards on the glass slides and in the data sheet.

The prepared paraffin blocks were serially sectioned at 4 μm thickness and stained with Hematoxylin and Eosin (H&E) stains for histopathological diagnosis. The malignant cases were further examined for Gleason grading and detection of perineural invasion. Each case was assigned a Grade group according to latest WHO recommendations (Netto et al., 2022).

Immunohistochemical Staining

For immunostaining, two additional sections on positive charged slides were prepared from each paraffin block. Immunostaining was performed using a Dako Omnis immunostainer. An anti- AMACR (13H4) Rabbit IGg monoclonal antibody (#IR060: Agilent; USA) and anti-ERG (EP111) Rabbit IGg monoclonal antibody (#IR659: Agilent; USA) were used.

P63 immunostaining using an anti-P63 (DAK-P63) mouse monoclonal antibody (#IR662: Agilent; USA) was used when needed for better stratification of few cases that showed suspicious foci on H&E sections (Figure 1).

Immunohistochemical Evaluation

Evaluation of immunohistochemical staining was performed by 2 pathologists. Cytoplasmic staining (for AMACR) and nuclear staining (for ERG) were evaluated quantitatively and qualitatively. Intensity of staining was scored into no, weak, moderate and strong staining while percentage of immunoreactive cells was scored as continuous variable.

For each case, H-score was calculated for each marker using the following formula: (3 x percentage of strongly staining cells) + (2 x percentage of moderately staining cells) + (1 x percentage of weakly staining cells) + (0 x percentage of negatively staining cells) to get a score ranging from 0 to 300. For further analysis, Cases with ≤10 score was categorized as no expression, 11–100 as low expression, 101–200 as intermediate expression, while >200 score was taken as high expression (Hashmi et al., 2019).

Statistical analysis

For statistical analysis, SPSS statistical software program version 26 (SPSS Inc., Chicago, IL, USA.) was used. Data was expressed as frequencies and percentages. Chi square χ^2 test, Fisher's Exact Test or Pearson Monte Carlo test were used when applicable for comparing qualitative variables. Specificity, sensitivity, negative predictive value (NPV), and positive predictive value

(PPV) were calculated for AMACR and ERG considering the histopathologic diagnosis as the gold standard test. The receiver operating characteristic (Roc) curve was carried out for AMACR and ERG expressions and areas under the curves (AUC) were estimated with its 95% confidence interval (CI). All tests were two sided; P value is considered significant if < 0.05.

Slides Screening And Imaging

All slides were examined using an Olympus light microscope (model BX53F2). Images were obtained by digital Olympus high-definition camera (model EP50) connected to the same microscope.

Results

This study included 22 cases of prostatic hyperplasia and 50 cases of prostatic carcinoma. Eleven of our cases aged less than 60 and 61 cases aged 60 or more. Regarding the grade groups of the malignant cases; 4 cases were grade group 1; 5 cases were grade group 2; 11 cases were grade group 3; 16 cases were grade group 4 and 14 cases were grade group 5. Peri-neural invasion was detected in 23 (46%) of the malignant cases. The clinico-pathological parameters of the cases are presented in Table 1.

AMACR immunohistochemical expression was detected in 82% of the malignant cases with variable degrees of expression (low, intermediate and high), it was also detected in 45.4% of benign cases. AMACR expression correlated significantly with the type of prostatic lesion (benign versus malignant) (p = 0.001), as well as, the age of patient (p = 0.007), grade groups of carcinoma cases (p = 0.006) and the presence of perineural invasion (p = 0.011) (Table 1, Figures 2&3).

As for ERG expression, it was not detected in any of the benign cases and detected only in 22% of the malignant cases, however, no statistical significance was detected when studying differential ERG expression in benign and

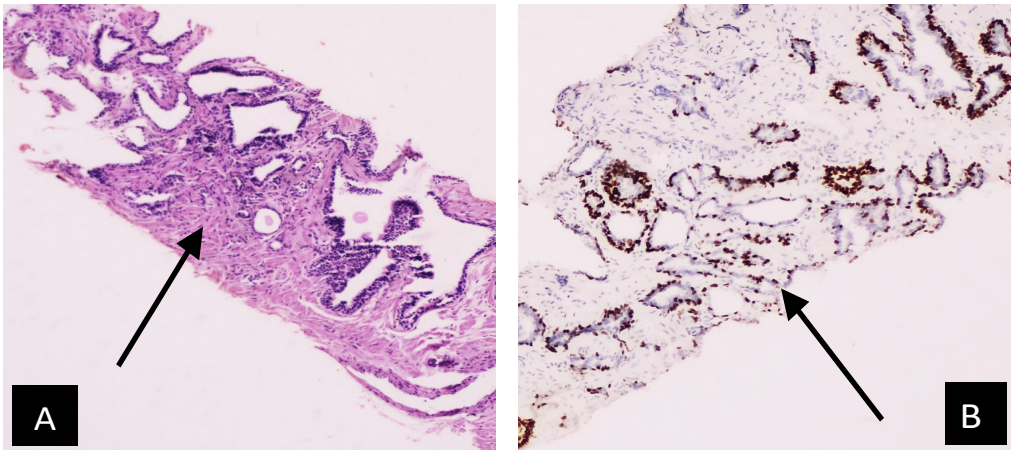

Figure 1. A) Case with suspicious small glands on H&E examination (arrow). B) P63 staining on serial sections highlighting the basal cell layer of the glands.

Table 1. Association of AMACR and ERG Expression With the Clinicopathological Data of the Studied Cases

Parameters	Total	AMACR Expression number (%)				p value¥	ERG Expression number (%)				p value¥
		No expression (n=16)	Low expression (n=22)	Intermediate expression (n=23)	High expression (n=11)		No expression (n=61)	Low expression (n=3)	Intermediate expression (n=1)	High expression (n=7)	
Age groups (years)						0.007					0.397
<60	11	6 (54.5)	2 (18.2)	0 (0)	3 (27.3)		11 (100)	0 (0)	0 (0)	0 (0)	
>=60	61	10 (16.4)	20 (32.8)	23 (37.7)	8 (13.1)		50 (82)	3 (4.9)	1 (1.6)	7 (11.5)	
Type of prostatic lesion						0.001					0.087
Benign	22	12 (54.4)	7 (31.8)	3 (13.6)	0 (0.0)		22 (100)	0 (0)	0 (0)	0 (0)	
Malignant	50	4 (8)	15 (30)	20 (40)	11 (22)		39 (78)	3 (6)	1 (2)	7 (14)	
Grade (n=50)						0.006					0.044
Grade I	4	2 (50)	0 (0)	2 (50)	0 (0)		3 (75)	0 (0)	0 (0)	1 (25)	
Grade II	5	0 (0)	2 (40)	2 (40)	1 (20)		2 (40)	2 (40)	0 (0)	1 (20)	
Grade III	11	1 (9.1)	6 (54.5)	2 (18.2)	2 (18.2)		9 (81.8)	0 (0)	1 (9.1)	1 (9.1)	
Grade IV	16	0 (0)	0 (0)	10 (62.5)	6 (37.5)		15 (93.8)	1 (6.3)	0 (0)	0 (0)	
Grade V	14	1 (7.1)	7 (50)	4 (28.6)	2 (14.3)		10 (71.4)	0 (0)	0 (0)	4 (28.6)	
Perineural invasion (n=50)						0.011					0.183
Absent	27	3 (11.1)	7 (25.9)	14 (51.9)	3 (11.1)		18 (66.7)	2 (7.4)	1 (3.7)	6 (22.2)	
Present	23	1 (4.3)	8 (34.8)	6 (26.1)	8 (34.8)		21 (91.3)	1 (4.3)	0 (0)	1 (4.3)	

¥, Monte Carlo test was applied as 25.0% or more cells have expected count less than 5.; p-value<0.05 considered as significant.

Table 2. Association between AMACR and ERG Expressions in Prostatic Lesions

ERG Expression	Total	AMACR Expression n (%)				p value¥
		No expression (n=16)	Low expression (n=22)	Intermediate expression (n=23)	High expression (n=11)	
No Expression	61	16 (26.2)	15 (24.6)	19 (31.1)	11 (18.0)	
Low Expression	3	0 (0.0)	1 (33.3)	2 (66.7)	0 (0.0)	P=0.151¥
Intermediate Expression	1	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	
High Expression	7	0 (0.0)	5 (71.4)	2 (28.6)	0 (0.0)	

¥, Monte Carlo test was applied as 25.0% or more cells have expected count less than 5; AMACR, Alpha-methylacyl- CoA coenzyme A racemase; p-value<0.05, considered as significant.

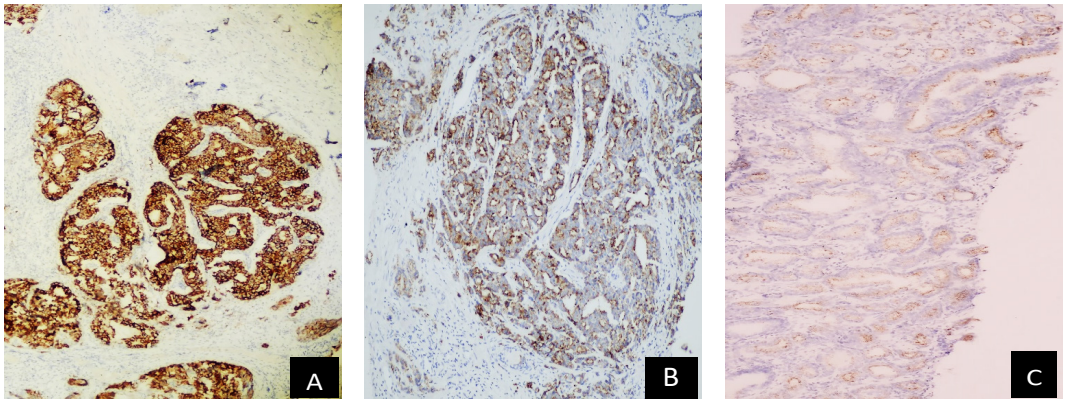

Figure 2. AMACR Immunohistochemical Expression in Prostatic Carcinoma Cases; considered as strong (A), moderate (B) and weak (C) intensities.

malignant cases (p = 0.087). In malignant cases, ERG expression correlated significantly with the grade groups (p = 0.044) (Table 1, Figure 4).

No statistically significant correlation was detected between AMACR and ERG expression in our study (p = 0.202) (Table 2). Representative cases of both AMACR and ERG expression; one benign (Figure 5) and one malignant (Figure 6) are presented.

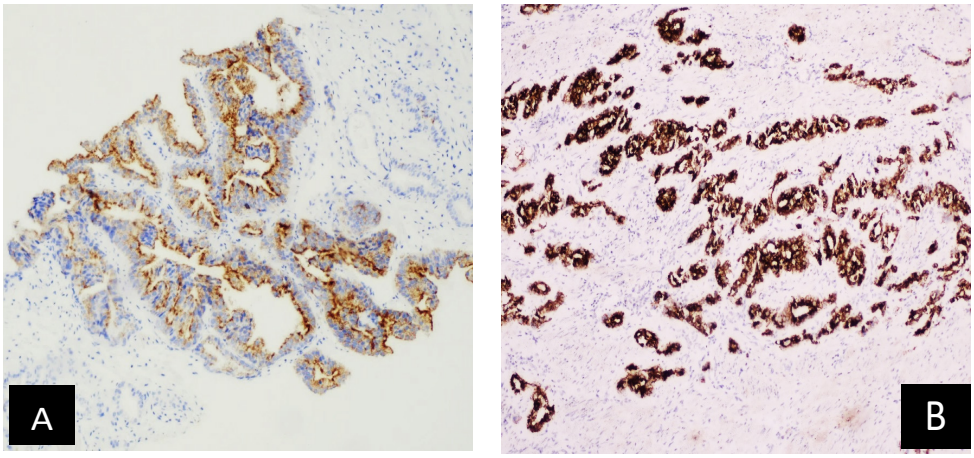

Figure 3. AMACR IHC Expression in Prostatic Hyperplasia (A) and Prostatic Carcinoma (B)

Table 3. The Diagnostic Validity of AMACR and ERG Expression in Differentiating benign and Malignant Prostatic Lesions

Test	AUC	95% Confidence Interval	Kappa value	P- value	Cut off point	Sensitivity	Specificity	PPV	NPV	Accuracy
AMACR	0.88	0.81 -0.96	0.4	<0.001	95	62	86.4	91.2	50	69.4
ERG	0.61	0.50-0.74	0.15	0.102	30	22	100	100	36.1	47.8
Combined	0.81	0.71 -0.92	0.49	<0.001	----------	76	86.4	92.7	61.3	79.2

AUC, Area under the receiver operating characteristic (ROC) curve; PPV, positive predictive value; NPV, negative predictive value. A guide for classifying the accuracy

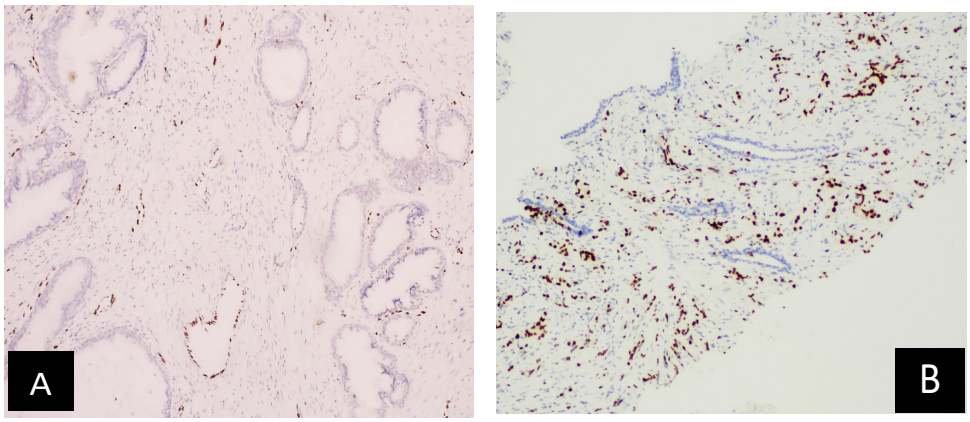

Figure 4. A) Negative ERG IHC expression in Prostatic Hyperplasia with positive internal control (vessels). B) Positive ERG IHC expression in prostatic carcinoma with negative intervening benign glands.

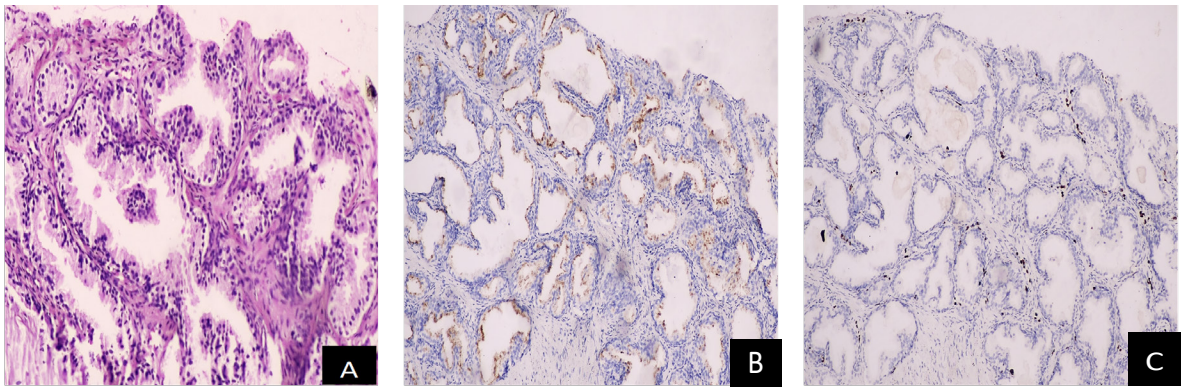

Figure 5.A case of Prostatic Hyperplasia; A) H&E stain, B) Positive weak to moderate AMACR IHC expression. C) Negative ERG IHC expression with positive internal control (vessels)

Regarding the diagnostic validity of AMACR and ERG expressions in differentiating benign and malignant prostatic lesions (Table 3, Figure 7), AMACR showed statistically significant validity (p = <0.001) with 86% Sensitivity and 62% specificity for malignant lesions. Although ERG showed 100% specificity for malignant lesions, its sensitivity for detecting malignant lesions was only 22% (p = 0.102). Combining both markers led to higher diagnostic accuracy (79.2) compared to using AMACR alone (69.4) or ERG alone (47.8) (Table 3).

Discussion

Given the high incidence of PC and the fact that histopathologic diagnosis of some prostatic lesions may be challenging, using immunohistochemical markers is crucial for the diagnosis of some cases. Basal cell markers such as P63 are among the most commonly used markers in such cases. To increase the specificity of a carcinoma diagnosis further beyond basal cell immunohistochemistry, AMACR has been the first marker to be used in common practice, after its identification as

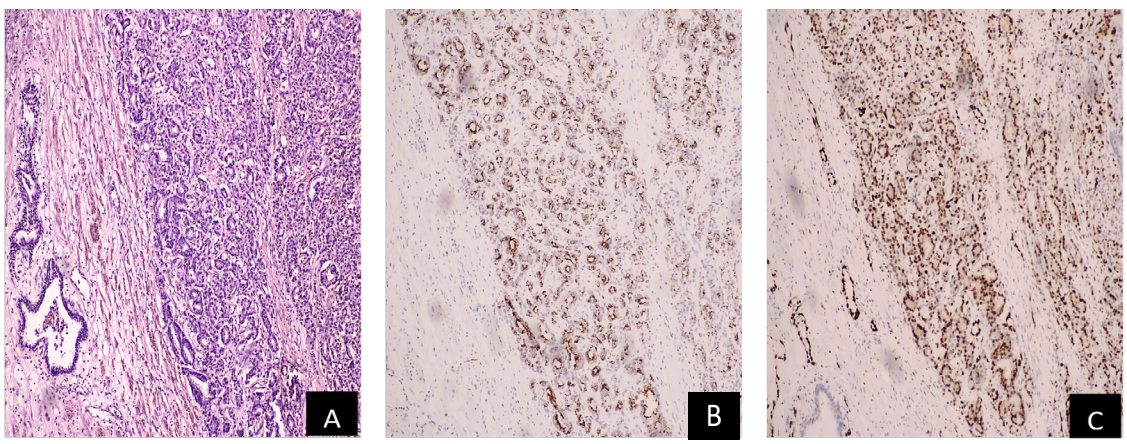

Figure 6. A Case of Prostatic Carcinoma; A) H&E stain, B) Positive AMACR IHC expression. C) Positive ERG IHC expression.

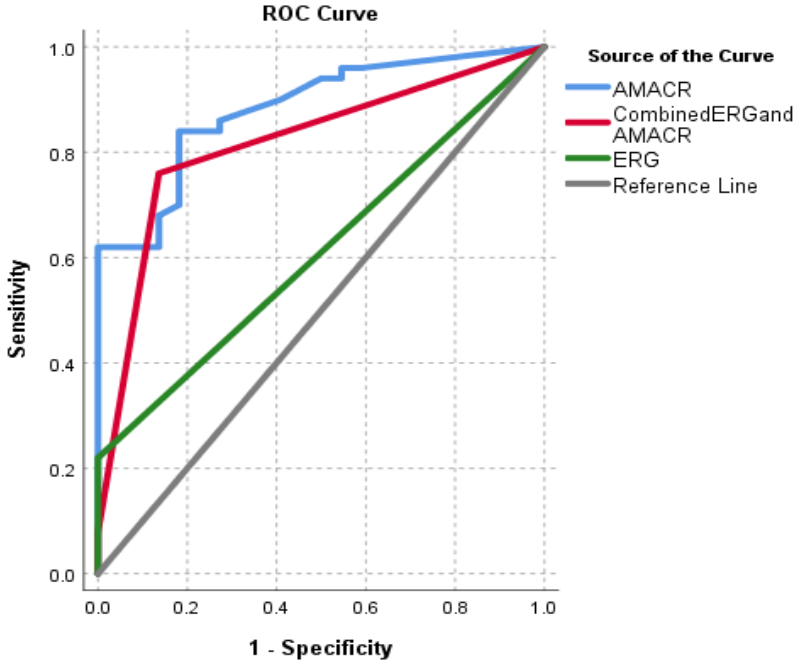

Figure 7.Receiver Operating Characteristic (ROC) Curve for the Diagnostic Validity of AMACR, ERG and Combined ERG and AMACR in Differentiating benign and Malignant Prostatic Lesions.

a commonly overexpressed transcript in PC (Kristiansen et al., 2018).

In fact, additional markers may be still needed for the diagnosis of some cases, owing to some limitations of basal cell markers and AMACR. Basal cell markers are sometimes discontinuous or patchy in benign glands (Hasan et al., 2020), so examination of more than one immunostained serial sections may be needed to reach accurate diagnosis. Regarding AMACR, despite its high sensitivity in staining malignant lesions, its expression in PC is heterogenous. Additionally, it may display weak to moderate expression in some benign lesions (Kristiansen et al., 2018).

TMPRSS2-ERG gene fusion is common in PC patients; immunohistochemistry using anti-ERG antibody showed excellent correlation with ERG rearrangement as determined by FISH (Hoogland et al., 2012). In this study, we investigated the immunohistochemical expression of AMACR and ERG in 50 cases of prostatic carcinoma using 22 cases of prostatic hyperplasia as controls to compare the validity of both markers in diagnosis of prostatic lesions.

In our study, AMACR showed statistically significant higher expression in prostatic carcinoma cases compared to prostatic hyperplasia, consistent with its widely accepted role in differentiating benign and malignant prostatic lesions (Box et al., 2016; Gouda and Eloseily, 2019). Although 54.5% of our benign cases were negative for AMACR, positive (low and intermediate) expression was detected in (31.8% and 13.9%) of our hyperplasia cases respectively. These figures are relatively higher than most of the reported figures in the literature, where the rate of AMACR positivity in benign prostatic lesions in some studies was 5.26% (Biswas and Talukdar, 2019), 7% (Yilmaz et al., 2019), 7.1% (Hasan et al., 2020) and 36.2% (Shafek, 2015). Despite the fact that many studies reported some degree of AMACR expression in benign prostatic lesions, others reported complete negativity of AMACR in benign cases (Gouda and Eloseily, 2019; Rathod et al., 2019; Nomani et al., 2021; Gudeli et al., 2021).

In our carcinoma cases, AMACR expression was completely negative in 8% and it showed (low, intermediate and high) expression in (30%, 40% and 22%) malignant cases respectively. The rate of AMACR positivity in our carcinoma cases is compatible with many studies in the literature reporting AMACR positivity in 87.7% (Bachurska et al., 2017), 89.6% (Shafek, 2015), 90% (Rathod et al., 2019), 92% (Hasan et al., 2020) and 95.8% (Stephen and Badhe, 2022). However, other studies reported 100% positivity of AMACR in prostatic carcinoma cases (Gudeli et al., 2021; Sandeep et al., 2021). These differences may be related to sample size, used anti-AMACR antibody clone and the cut off values used for interpreting AMACR staining as positive.

In our study, AMACR showed relatively heterogenous expression in carcinoma cases with only 22% considered to have strong expression. This result is compatible with the results of other reports scoring AMACR expression in carcinoma cases; Shafek (2015) reported 29.3% weak, 29.3% moderate and 31% strong AMACR expression in carcinoma cases. Kars (2020) similarly reported 13% score 1, 58.7% Score 2 cases and only 28.3% score 3 cases. Hasan (2020) reported Score 1, 2 and 3 AMACR expression in 10%, 30% and 50% of their cases respectively.

Other studies reported relatively stronger more diffuse AMACR staining in carcinoma cases; Stephan (2022) reported 60.6% Score 3 and 35.7% score 2 AMACR expression in their carcinoma cases. Also, Gudeli (2021); Sandeep (2021); Nomani (2021) reported that none of their malignant cases showed neither weak expression, nor very focal (<10% of tumor cells) positivity. Despite the

variable figures, most of the studies reported heterogenous AMACR expression in prostatic carcinoma cases in regard of the staining intensity and percentage of positive cells. A finding that sets a limitation when using AMACR to detect tiny foci of carcinoma in prostatic core biopsies.

A statistically significant association between AMACR expression and grade group was detected in our study with the highest rate of expression detected in grade group 4 cases (no negative or weak cases and 37.5% high expression cases). Grade 1 cases showed the lowest rate of expression with 50% negative cases and no detected high expression cases. The relation of AMACR expression and PC grade shows some debate in the literature. While some reports showed statistically significant higher expression in high grade cases (Gouda and Eloseily, 2019; Rathod et al., 2019), others showed higher expression in low grade cases (Shafek, 2015). Some other studies also showed no relation of AMACR expression to PC grade (Box et al., 2016; Kars et al., 2020; Stephen and Badhe, 2022).

According to our results, we agreed that AMACR expression in PC may have a prognostic value, being significantly expressed in higher grade cases and thus, it can be used as a therapeutic target (Yevglevskis et al., 2019). However, from the diagnostic point of view, the low expression of AMACR in low grade cases, which are the most likely to be confused with benign mimics, sets another limitation to the value of AMACR in differentiating benign from malignant prostatic lesions.

No statistically significant association was found between AMACR expression and perineural invasion in our study, however, higher rate of expression was found in cases positive for perineural invasion. This was consistent with the results of other studies (Gouda and Eloseily, 2019, Taheri et al., 2021).

Regarding ERG, no expression was detected in our benign cases; a finding showing wide agreement in the literature (Kristiansen et al., 2018). In our carcinoma cases, 78% were negative for ERG and 22 % showed variable degrees of expression; 6% low, 2% intermediate and 14 % high expression. Variable rates of ERG expression, yet mostly higher than ours, have been reported in the literature ranging from 70% (Gouda and Eloseily, 2019), 60% (Ibrahim et al., 2019), 55% (Hoogland et al., 2012), 51.8% (Dawoud et al., 2021), 35.2% (Stephen and Badhe, 2022), 33% (Falzarano et al., 2011) and 28.2 % (Bismar et al., 2018). Nie (2019) reported only 16.7 % positive ERG expression among their prostatic carcinoma cases. Unfortunately, in our study, no statistically significant difference was detected between ERG expression in hyperplasia and carcinoma cases.

Regarding the heterogenicity of ERG expression in carcinoma cases, although 63.7% of our positive carcinoma cases showed strong diffuse expression, 27.3% and 9% of the cases showed low and intermediate expression respectively. This was consistent with the results of other studies (Lee et al., 2015; Nie et al., 2019). Dawoud (2021) also reported similar results, yet with higher level of heterogenicity.

In our study, a statistically significant direct correlation was detected between ERG expression and Gleason grade. This was compatible with the results of some studies (Bismar et al., 2018; Gouda and Eloseily, 2019), yet, others reported inverted relation of ERG expression with grade group (Lee et al., 2015; Bismar et al., 2018; Ibrahim et al., 2019; Nie et al., 2019; Stephen and Badhe, 2022).

No significant association was detected between ERG and AMACR expression in our studied cases, keeping with what was reported by Box (2016); Gülhan (2020). However, another study reported a highly significant association between ERG and AMACR expression in prostatic lesions (Gouda and Eloseily, 2019).

On comparing the diagnostic value of AMACR and ERG in our study, ERG showed 100% specificity, yet, with much lower sensitivity than AMACR in differentiating benign from malignant prostatic lesions. The sensitivity, specificity, positive predictive value and negative predictive value were (22%, 100%, 100 and 36.1) respectively for ERG and (62%, 86.4%, 91.2 and 50) respectively for AMACR. This was compatible with the results of some studies (Lee et al., 2015; Stephen and Badhe, 2022). Gouda (2019) reported equal specificity for both markers with higher AMACR sensitivity for PC detection. On calculating the diagnostic accuracy, AMACR showed higher accuracy than ERG (69.4 versus 47.8), yet, combining both markers yielded better diagnostic accuracy (79.2) than AMACR alone owing to the higher specificity of ERG.

This led us to a conclusion that adding ERG as a second line immunohistochemistry in the diagnosis of histologically suspicious, yet AMACR negative, cases may be a valuable practice. This agreed with the conclusion of some (Bachurska et al., 2017; Stephen and Badhe, 2022), yet, others reported no additional diagnostic value of combining ERG with AMACR compared with AMACR alone (Andrews et al., 2014; Gouda and Eloseily, 2019). One limitation of this study is the lack of correlation with the patient's prognosis and survival.

Author Contribution Statement

Dr. Rasha Mohamed Samir Sayed: Study design, Literature search, immunostaining, slides examination, data analysis and statistical analysis. Dr. Galal El Shorbagy: Cases collection and data acquisition. Dr. Passant Essam Eldin Shibel: Literature search, slides examination and manuscript preparation.

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This work was approved by the scientific committee of the pathology department, faculty of medicine and also by the research ethics committee (REC) at faculty of medicine, Cairo university (REC code: N-80-2022).

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