



ISSN (P): 2617-7226
ISSN (E): 2617-7234
www.patholjournal.com
2020; 3(1): 396-403
Received: 09-11-2019
Accepted: 13-12-2019

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Utility of PIN4 cocktail antibody in the atypical foci of the Prostate

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DOI: <https://doi.org/10.33545/pathol.2020.v3.i1f.204>

Abstract

Aims: Diagnosis of prostatic carcinoma is based on histomorphological features. However, sometimes this can be challenging. In such situations immunohistochemistry can be a useful adjunct. This study is aimed to evaluate such suspicious or atypical cases by using PIN4 cocktail antibody (AMACR+p63+CK5/14).

Materials and methods: The present study was prospectively conducted at Upgraded Department of Pathology, Osmania general hospital, Hyderabad, Telangana state, India from August 2013 to July 2016. All hematoxylin and Eosin slides of prostatic specimens received during this period were routinely analyzed and cases with suspicious foci were subjected to immunohistochemistry. Immunohistochemistry was performed to look for expression of AMACR/p63/CK5/14 by using PIN4 Cocktail antibody.

Observation and results: Out of 306 prostatic specimens, 210 cases were Benign, 36 were malignant, 16 were PIN and 44 cases showed suspicious foci and these cases subjected to PIN4 cocktail antibody. Cases with p63+/CK5/14+/AMACR- were considered as benign, cases with p63-/CK5/14-/AMACR+ were considered as malignant, cases with p63+/CK5+/CK14+/AMACR+ were considered as Prostatic intraepithelial neoplasia (PIN)/Atypical adenomatous hyperplasia(AAH), cases with p63-/CK5/14-/AMACR- were considered as Atypical small acinar proliferation (ASAP).

Conclusion: Immunohistochemistry can be useful adjunct to biopsy. It reduces the chance of overdiagnosis of benign lesions as malignant, and under diagnosis of malignancies as benign. Also reduces the chances of unnecessary re-biopsy.

Keywords: PIN4 cocktail, atypical foci, prostatic carcinoma

Introduction

Prostate cancer is the second most common malignancy (next to lung cancer) in men worldwide and accounts for 3.8% cancer related deaths in men in the year 2018 [1, 2]. Prostate cancer incidence increases with age. Incidence of prostate cancer is highly variable worldwide. This variation might be because of extensive serum PSA testing in few countries [3]. Screening tests like serum PSA levels, DRE (Digital rectal examination), and imaging tests as MRI (especially Multiparametric MRI) or Transrectal ultrasound (TRUS) are helpful in early detection of prostate cancer. Any abnormality in one of these tests will need a prostate biopsy to exclude or confirm the diagnosis of prostate cancer. Histopathological examination of prostatic specimen is the standard test in arriving at a final diagnosis. Gleason grading system is given based on the microscopic appearance of prostate biopsy specimen and it is used in evaluating the prognosis of prostate cancer patients [4].

Histomorphology alone may fail in arriving at a diagnosis of ambiguous lesions especially when cancerous tissue is admixed with benign acini or due to presence of benign mimickers of carcinoma like reactive atypia, radiation atypia, clear cell cribriform hyperplasia, atrophy, basal cell hyperplasia, post atrophic hyperplasia, nephrogenic adenoma, cowpers glands, adenosis, squamous metaplasia [5]. Underdiagnosis of a small foci of malignant lesions or overdiagnosis of a cancer mimicking benign lesions can occur and can cause adverse effects for patients. Hence in such ambiguous cases, Immunohistochemistry helps in solving ambiguity and avoids underdiagnosis or overdiagnosis.

For the evaluation of cases with atypical/suspicious foci, a-methyacyl- CoA rasecase (AMACR) is usually used as a cancer-associated positive marker [6, 7]. Additionally, high

molecular weight cytokeratin (HMWCK), cytokeratin 5/6 and p63 are generally employed as markers of basal cells (negative markers) [6, 8]. In this study we looked for AMACR, p63, K5/14 expression by using PIN4 cocktail antibody with double chromogen.

PIN4 COCKTAIL ANTIBODY consists of combination of P504S(AMACR), HMW CKs (CK5 /14) & p63, Clone-XM26+LL002+BC4A4+N/A (from Biocare USA, ready to use). P504S (AMACR) shows cytoplasmic (red) positivity, p63 shows nuclear (brown) positivity, HMW CKs show cytoplasmic (brown) positivity.

AMACR: AMACR gene is located on 5p13.2. This gene encodes alpha-methylacyl-CoA racemase enzyme. It is found in mitochondria and peroxisomes. It is involved in beta-oxidation of branched chain fatty acids, and fatty acid derivatives (including bile acid intermediates). Its deficiency in adults causes neurological disorders, in infants it also causes congenital bile acid synthesis defect [9, 10]

Immunohistochemical expression

AMACR immunoreactivity is cytoplasmic. It is normally expressed in renal proximal tubular cells, hepatocytes. It is predominantly expressed in prostatic glandular cells in adenocarcinoma, Prostatic intraepithelial neoplasia, hepatocellular carcinoma, renal cell carcinoma. Variable expression is seen in lymphomas, carcinomas of breast, ovary, and lung. It is not or weakly expressed in normal prostate glands [11]

p63: p63 is a member of the p53 gene family. This gene is located on chromosome 3q27-29. It encodes at least six different transcripts with transactivation (Tap63) or negative effects (Np63) on the p53 reporter genes, resulting in tumor suppressor and oncogenic effects respectively. During embryogenesis, it is essential for the development of several epithelia. Human germ line mutations result in limb mammary syndrome (ectrodactyly, ectodermal and facial clefts) with hypoplasia/aplasia of the breasts. p63 (-/-) mice do not develop prostate [12]. It is a sensitive and specific marker of myoepithelial cells.

Immunohistochemical expression – p63 immunoreactivity is nuclear. In normal tissues, expression is noted in stratified epithelial cells like skin, tonsil, esophagus, ectocervix & bladder and also in the glandular structure basal cells of breast, prostate and bronchi. In respiratory tract, goblet cells along with normal ciliated cells are negative for p63, but reserve cells are positive for p63.

p63 is expressed in squamous cell, basal cell, and transitional cell carcinoma, but not in adenocarcinomas, including those of breast and prostate. A subset of Non Hodgkin lymphoma express p63.

CK5 /14: CK5 and CK14 are proteins encoded by KRT5 and KRT14 genes which are located on chromosome 12q13.13 and 17q21.2. Keratin 5 partners with a similar protein, keratin 14, to form molecules called keratin intermediate filament. Both CK 5 and CK14 are high molecular weight cytokeratin. These filaments assemble into strong networks that help attach keratinocytes together and anchor the epidermis to underlying layers of skin. The network of keratin intermediate filaments provides strength

and resiliency to the skin and protects it from being damaged. Keratin 5 deficiency leads to Dowling-Degos disease which is characterised by hyperpigmentation, most often body folds and creases. Mutation in KRT5 and KRT14 genes leads to epidermolysis bullosa simplex, a condition that causes the skin to be very fragile and to blister easily [13].

Immunoeexpression: CK 5 and CK14 are normally expressed in breast myoepithelial cells, cornea, mesothelium, nail unit of adult, prostate basal cells, skin (basal layer), salivary glands (basal layer), squamous epithelium (basal cells), urothelium (basal cells). In an adenomatous malignant tumour, expression of the stratified-epithelial CK5 (and CK14) may be suggestive of pancreatic-biliary adenocarcinoma, ovarian serous or endometrioid carcinoma, adenosquamous carcinoma, breast stem cell carcinoma or myoepithelial carcinoma or, in serosal biopsies, malignant mesothelioma. Adenocarcinomas of lung and breast rarely show CK5 expression. In squamous cell carcinoma there is increased expression of CK5 and CK14. CK5 and CK14 expression is lost in prostate adenocarcinoma [14].

The limitation of using only a negative marker for diagnosis of the carcinoma is that basal cells can be patchy or discontinuous in some benign lesions. Using AMACR as a positive marker alone might be misleading, because weak expression of AMACR might also be seen in benign glands, and expression of AMACR is not only seen in prostatic adenocarcinoma but also in highgrade prostatic intraepithelial neoplasia (PIN) [15, 16] and atypical adenomatous hyperplasia (AAH) [17]. Therefore, using AMACR as a positive marker in combination with basal cell-specific negative markers (HMWCK, p63) will help in arriving at a diagnosis in atypical foci of prostate.

Various antibody cocktails have been investigated for the diagnosis of prostatic cancer in needle biopsies, transurethral resections and prostatectomies [18-26]. We used PIN4 cocktail antibody (AMACR +p63+Cytokeratin 5/14) in reaching a definite diagnosis of suspicious foci.

Materials and methods

All prostatic specimens collected in Upgraded department of pathology in Osmania general hospital, Hyderabad over a period of 36 months (august 2013 to July 2016) were routinely processed and analyzed. The age of the patients in our study ranged from 34 years to 92 years. H&E-stained slides of 306 cases were examined thoroughly and a provisional diagnosis of each case was made and they were divided into four categories- benign (210), PIN (16), cases with suspicious foci (44) and malignant (36). Suspicious cases included cases with small crowded glands which are showing some, but not all architectural and cytological features of adenocarcinoma. In some cases, these glands are seen in <5% of the biopsy, raising the suspicion of benign mimickers of carcinoma. In few cases, there is an ambiguity in diagnosing whether it is a cribriform carcinoma or cribriform PIN. The serum PSA levels in these suspicious cases ranged from 03 to 72.6ng /dl. Cases with suspicious foci were subjected to immunohistochemistry in arriving at a definitive diagnosis. In this study PIN4 cocktail antibody (AMACR+p63+CK5/14) was used.

Table 1: Pin4 Cocktail Antibody

Antibody	Amacr/P504S	P63	CK5	CK14
Clone	pAb	BC4A4 (mAb)	XM26 (mAb)	LL002 (mAb)

(pAb-polyclonal antibody, mAb- monoclonal antibody)

It's a Biocare product (Ready to use)

Positive control is Prostatic intraepithelial neoplasia(PIN)

Staining Protocol

- The sections taken from the paraffin blocks should have to be kept for drying for atleast 1-2 hrs.
- Slides are kept in Hot air oven at 75 degree centigrade for 20 min
- Deparaffinization of slides
- Antigen retrieval (100 degree for 25 mins in EDTA with Ph 7.4 to 7.6).
- Hydrogen peroxide (H2O2) for 10 min.
- Protein block solution for 10 min.
- Primary antibody ('PIN 4 cocktail antibody') for 45 min.
- Secondary antibody ('MACH 2 Double stain') for 45 mins.
- 'DAB' (Diamino benzidine) for 8min.
- 'Wrap Red' for 10min.
- Counterstain with hematoxylin for 1 Min.
- Above each successive step should be coupled with washing by using TBS/DW for 2min.

- Allow the slides to dry and then mount using DPX.

Table 2: Evaluation for Immunohistochemistry

Antibody	Immunoreactivity	Colour
AMACR	Cytoplasmic	Red
CK5/14	Cytoplasmic	Brown
P63	Nuclear	Brown

Amacr

AMACR staining is interpreted in accordance with Kumaresan *et al.* [5]. Positive staining means dark diffuse or granular, cytoplasmic or luminal, but circumferential. The percentage positivity was graded from 0+ to 3+ as follows

Table 3: Grading

0	When there is absolutely no staining observed.
1+	1-10% cells in a gland show Positive staining.
2+	11 – 50% cells in a gland show Positive staining
3+	> 50% cells in a gland show Positive staining

Negative staining means no staining or focal, weak noncircumferential fine granular staining.

Basal cell markers

CK5/14 is interpreted as cytoplasmic positive/negative, continuous and discontinuous.

P63 is interpreted as nuclear positive/negative, continuous and discontinuous.

Table 4: Immunohistochemical profile

	Staining pattern	Final Diagnosis
Basal cells	Luminal cells	
P63+/CK5/14+	P504S-	Benign
P63+/CK5/14+	P504S+	PIN (Prostatic Intra Epithelial Neoplasia) OR AAH (Atypical Adenomatous Hyperplasia)
P63-/CK5/14-	P504S-	Asap (Atypical Small Acinar Proliferation)
P63-/CK5/14-	P504S+	Prostatic Adenocarcinoma

Results

The present study constituted a total of 306 cases. All prostatic specimens were broadly classified into benign, prostatic intraepithelial neoplasia, malignant, and suspicious

lesions. The age distribution of these lesions is depicted in Table 5.

These lesions were further subdivided and details are depicted in the below tables 5, 6 &7 and figures.

Table 5: Age distribution

Age	Benign	Prostatic Intraepithelial Neoplasia (Pin)	Malignant	Suspicious Lesions
<50	18	00	02	04
51-60	34	03	05	14
61-70	86	07	11	12
71-80	54	04	15	07
81-90	17	02	03	07
>90	01	-	-	00
Total	210	16	36	44

The age of the patients in our study ranged from 34 years to 92 years; however, the predominant population was in the 6th to 7th decade with a mean age of 65.9years.

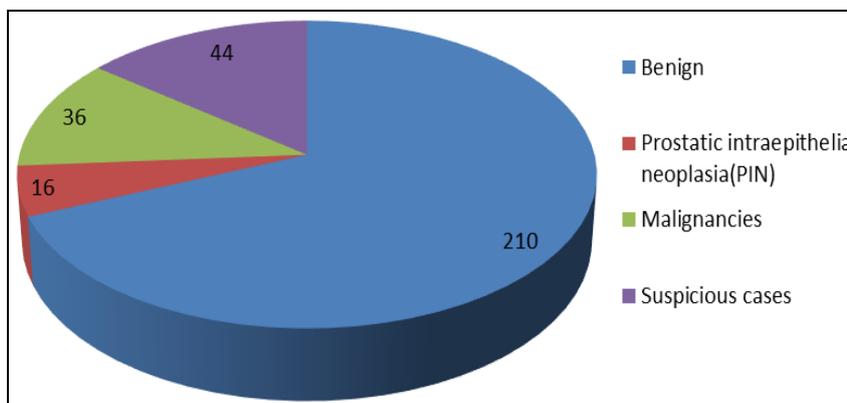


Fig 1: Distribution of cases

Out of total 306 cases, 210 (68.62%) cases were benign, 36 cases (11.76%) were malignant i.e, 31 were prostate adenocarcinoma cases and 05 cases were transitional cell carcinomas. PIN cases were 16 (5.22%) and suspicious cases were 44 (14.37%).

Table 4: Categorization of benign cases

Benign	Cases
BPH	116
BPH with acute and chronic prostatitis	83
Atrophy	06
Squamous metaplasia	04
Non-specific granulomatous prostatitis	01
Total cases	210

Table 5: Categorization of PIN cases

Prostatic intraepithelial neoplasia (PIN)	Cases
Lgpin	09
Hgpin	07

Table 6: Categorization of malignant cases

Malignant	Cases
Adenocarcinoma	31
Transitional cell carcinoma	05

Table 7: Type of specimen showing suspicious foci

Specimen	Cases
Transurethral resection of Prostate	30
Needle biopsy	14

Table 8: Final diagnosis of Suspicious lesions after immunohistochemistry

	Benign P63+/CK5/14+ AMACR-	Carcinoma P63-CK5/14- AMACR+	ASAP P63- /CK5/14- AMACR-	PIN (Prostatic intraepithelial neoplasia) P63+/CK5/14+ AMACR+
TURP specimens (30 cases)	13	10	05	01
Needle biopsy (14 cases)	03	08	03	01
Total	16	18	08	02

Immunohistochemistry was done on suspicious cases using PIN4 cocktail antibody. Out of 30 cases of TURP specimens with suspicious foci, 13 cases were diagnosed as benign, 10 cases were diagnosed as adenocarcinomas, 01 case diagnosed as PIN and 05 cases were diagnosed as ASAP

(Atypical small acinar proliferation). Out of 14 needle biopsies, 03 cases were benign, 08 cases were adenocarcinomas, 01 case diagnosed as PIN and 03 cases were diagnosed as atypical small acinar proliferation that required follow-up.

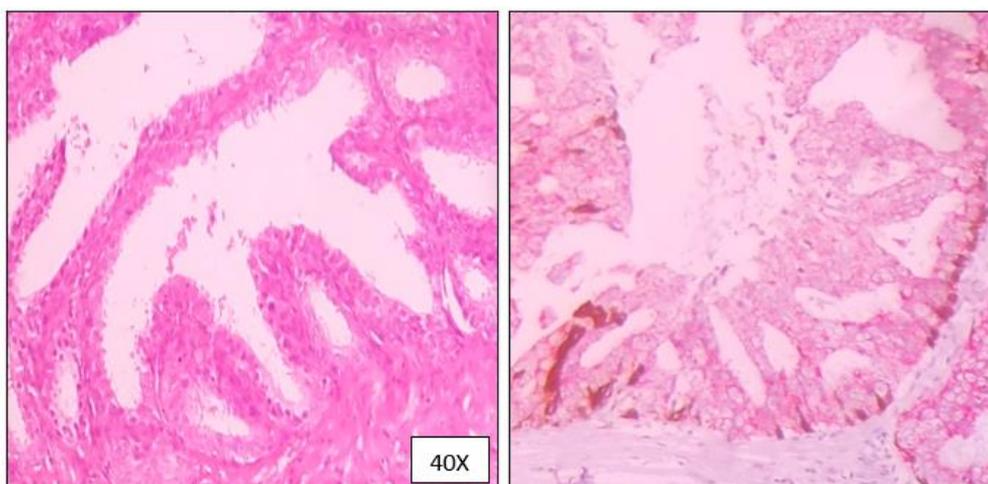
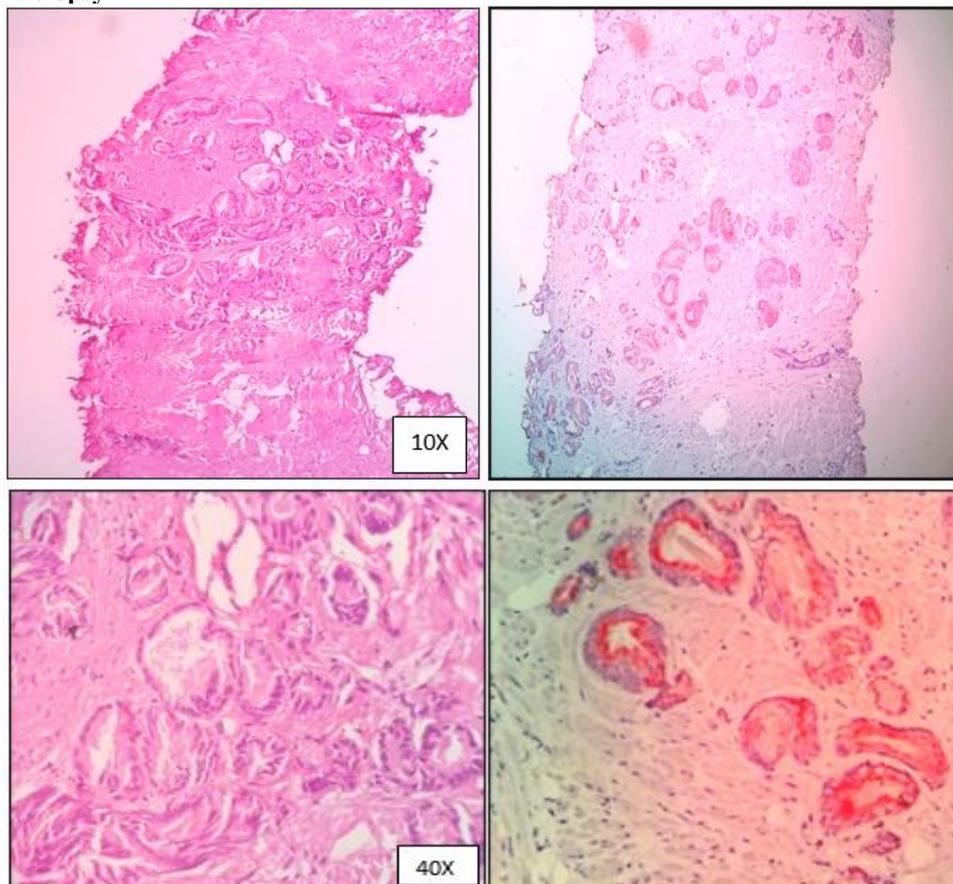


Fig 2: Suspicious foci diagnosed as Prostatic intraepithelial neoplasia on IHC (AMACR+, P63+, CK5/14+)

3A: Prostate needle biopsy



3B: TURP Specimen

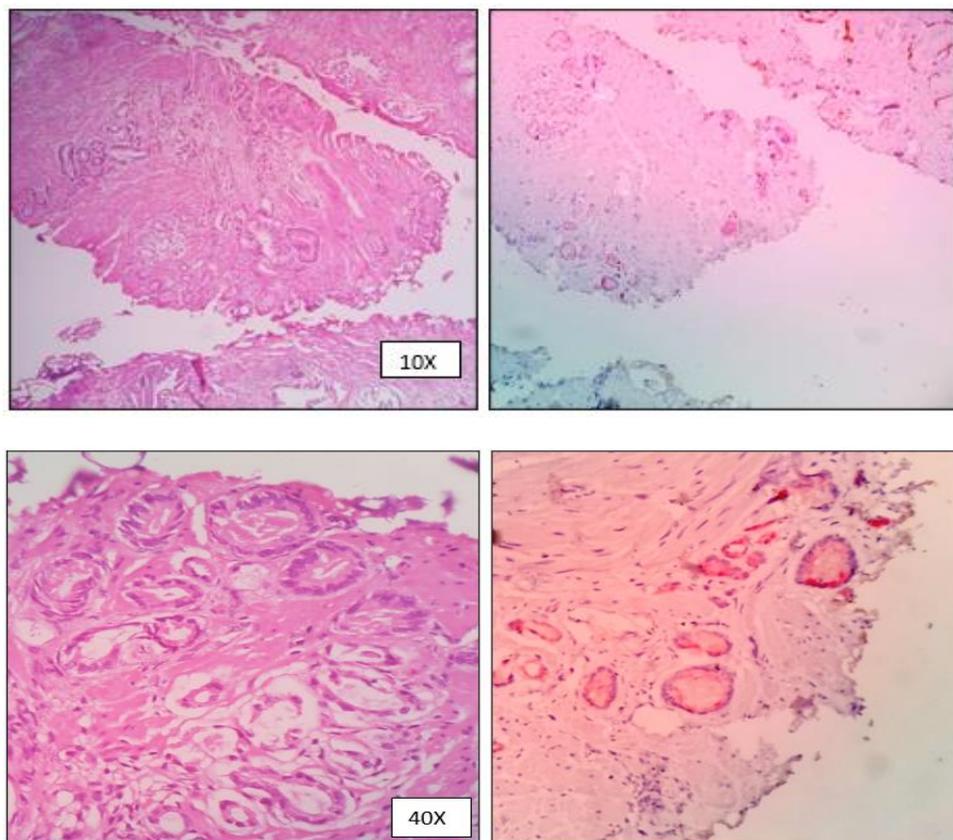


Fig 3A: and 3B: Suspicious foci diagnosed as adenocarcinomas on IHC (AMACR+,P63-,CK5/14-)

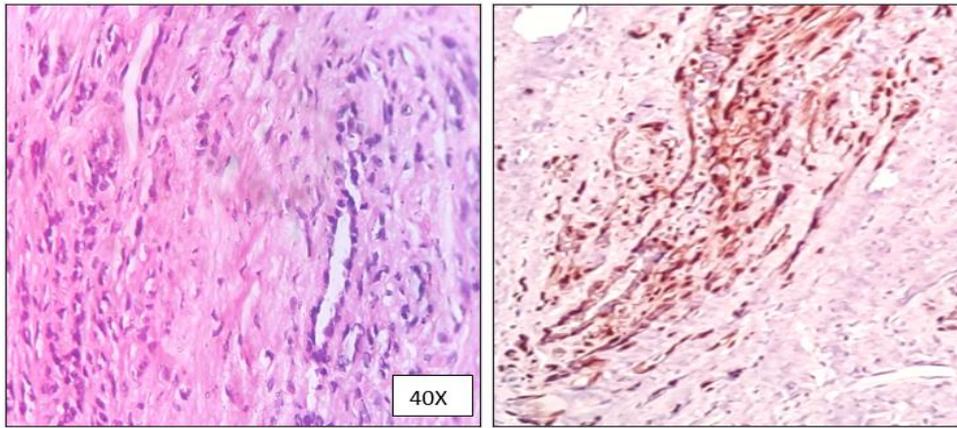


Fig 4: Suspicious cases diagnosed as Benign on IHC (AMACR-, P63+, CK5/14+)

Discussion

The age of the patients in our study ranged from 34 years to 92 years; however, the predominant population was in the 6th to 7th decade with a mean age of 65.9 years. The results of the present study agree with the studies by George and Thomas^[27], in which the mean age was 66.81 years, and by Barakzai *et al.*^[28], in which the mean age was 66.9 years.

In our study, most common benign lesion is benign prostatic hyperplasia constituting 68.6% of total cases, most common malignancy prostatic adenocarcinoma constituting 11.7% of total cases. These results were comparable with studies of Mittal *et al.*^[29], Anjorini *et al.*^[30].

When pathologists encounter a small amount of atypical cells or benign mimickers of carcinoma in prostatic specimens, ambiguity between lesions like cribriform PIN versus Cribriform carcinoma, a diagnostic difficulty occurs. In such circumstances, immunohistochemistry can be a powerful adjuvant for diagnosing prostatic cancer. On one hand, a new marker, α -methylacyl-CoA rasmase, has been identified and many pathologists use the antibody against AMACR in the diagnostic challenging cases as a positive marker^[6, 31]. On the other hand, antibodies against basal cell markers have traditionally been tried.

Previously, various cocktail antibodies including AMACR/p63, AMACR/p63/HMWCK or AMACR/p63/CK5/6 using one or two chromogens have been investigated by some researchers^[18-26].

In our study for the evaluation of suspicious foci, immunohistochemistry was performed to look for the expression of AMACR/p63/CK5/14 by using PIN4 cocktail antibody with double chromogen. Out of 44 cases with suspicious foci, 30 were TURP specimens and 14 were needle biopsies. Out of 44 cases, 16 cases (36.36%) were diagnosed as benign, 18 cases were diagnosed as adenocarcinoma (40.9%), 02 cases were diagnosed as PIN (4.54%) and 08 cases (18.1%) were diagnosed as Atypical small acinar proliferation (ASAP).

The suspicious lesions diagnosed as benign in our study might be cases of common benign mimickers of carcinoma like post atrophic hyperplasia or reactive atypia or atrophy. The suspicious lesions which were positive for AMACR/P504S and negative for basal cell markers were considered as adenocarcinoma. A negative expression of AMACR/P504S in small acini does not exclude a malignant diagnosis.

ASAP are either benign mimickers of carcinoma or

carcinoma which is marginally sampled. Suspicion of malignancy is high in these lesions and IHC might not be helpful in arriving at an exact diagnosis. Follow-up is advised in these lesions with serum PSA levels, digital rectal examination, Transrectal ultrasound for every 6 months.

Out of 08 cases of ASAP, 04 cases were followed up and 02 cases were turned out to be prostatic adenocarcinoma on repeat biopsies.

Rubin *et al.* found AMACR sensitivity and specificity to be 97% and 100% in detection of prostate cancer^[16]. Several previous studies have shown that small foci of carcinoma in the needle biopsy specimens express AMACR with 80% to 100% sensitivity^[18-20, 32].

Zhou *et al.*^[33] reported that basal cell cocktail (HMCK/p63) stains show the basal cell layers more intensely than p63 or HMWCK alone and benign glands lack basal cell lining in 02% of glands with cocktail staining. Consequently, HMWCK (clone-34bE12)/p63 cocktail staining not only increases the sensitivity of the basal cell detection but also reduces the staining variability and, therefore, provides more reliable immunostaining results^[33].

Immunohistochemical analysis with a triple-antibody cocktail is an assay with high specificity for prostate carcinoma^[32]. Although expression of AMACR might increase the confidence level in the definitive diagnosis of small focal prostate cancer, high-grade PIN, AAH, and some benign glands might exhibit some reactivity for AMACR. Because benign glands usually are lined by basal cells, the combination of AMACR/P504S and HMWCK or p63 can be used to recognize benign glands if both markers are positive in the same gland.

V Molinie *et al.*^[19] study showed that cocktail p63/P504s is more specific than CK5/6 alone. In 40% of atypical cases (which were previously diagnosed as ASAP), prostatic cancer diagnosis is established by using p63/P504s cocktail antibody^[19]. In our study, out of total suspicious cases, 40.9% were finally diagnosed as prostate adenocarcinomas. Jiang *et al.*^[32] study showed that AMACR is expressed in 95% of small focal carcinomas with triple-antibody cocktail staining. The positive rate of AMACR with the triple-antibody cocktail in immunohistochemical analysis is similar to that found in a previous study using single-antibody (P504S) staining^[18]. Their results showed that immunohistochemistry with a 3-antibodycocktail and double chromogen is a simple and easy assay compared to antibodies used individually.

Naota Koroda *et al.*^[34] used AMACR/p63 cocktail, CK5

and D2-40 in evaluating atypical lesions of prostate needle biopsies and a final diagnosis is made in majority of the cases.

In Hameed O *et al* study [20], the use of AMACR/basal cell antibody cocktails has been found to greatly facilitate the distinction between prostatic carcinoma and its benign mimickers. Ng *et al.* [23] have also reported that the specificity of three cocktail including AMACR/p63/HMWCK using one chromogen is superior to the antibodies used individually.

Dabir *et al.* have reported that the specificity of three antibody cocktail including AMACR/p63/CK5 using two chromogens is superior to that of two-antibody cocktail including AMACR/p63 using one chromogen [8].

We suggest that it may be difficult for pathologists to interpret cocktail antibody with single chromogen instead of double chromogen.

Bikash Sabata *et al* [36] compared automated analysis versus manual analysis of PIN4 stained prostate biopsies and they got 98% concordance. It shows manual analysis of PIN4 stained slides is good enough.

Epstein *et al* study [35] showed that 34 – 60% cases with atypical small acinar proliferation (ASAP) in the initial biopsies were later diagnosed as prostate carcinomas in the repeat biopsies. In our study, out of 08 ASAP cases, 50% cases were followed up and in that, half of the cases were found to be having prostate adenocarcinoma in the repeat biopsies.

Advantages of triple cocktail antibody with double chromogen over dual cocktail antibody and individual antibodies [18].

1. By using triple cocktail antibody, underdiagnosis and overdiagnosis of prostatic lesions can be avoided.
2. Immunohistochemistry by individual antibodies need repeated block cuttings which will result in the loss of small lesions and repeat biopsy will be required in such cases. These can be avoided by triple cocktail antibody.
3. Cocktail antibody with double chromogen is always superior to cocktail antibody with single chromogen as the interpretation will be better.

Limitations of cocktail antibody [18]

1. Sometimes, atypical small foci will be seen in prostate specimens as a result of tangential sectioning of HGPIN involved acini. In such cases, acini will be positive for P504S and negative for p63 as HGPIN will have discontinuous basal layer and finally resulting in overdiagnosis of the lesion as adenocarcinoma. This stain cannot help in differentiating adenocarcinoma with HGPIN from HGPIN alone.
2. Benign mimickers of adenocarcinoma like adenosis and AAH are P504S positive and they have a discontinuous basal layer. Limited sampling results in the misinterpretation of these lesions as adenocarcinoma. So, interpretation must be done cautiously.

Conclusion

PIN 4 cocktail antibody (AMACR/p63/CK5/14) is useful in evaluating atypical foci especially when the biopsy material is limited as application of individual antibodies causes loss of atypical glands by further sectioning. It is also suggested that this three-antibody cocktail with double chromogen can

reduce the chance of overdiagnosis of benign lesions as malignant, and underdiagnosis of carcinomas as benign and reduces the chances of repeat biopsy. Interpretation of PIN4 stained slides should be done cautiously.

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