



UTILITY OF P63 AND AMACR APPLIED AS DOUBLE SIMULTANEOUS IMMUNOSTAINS IN DIAGNOSTIC ASSESSMENT OF PROSTATE SPECIMENS

Dr Ajit Nambiar*

Associate Professor of Pathology, Amrita Institute of Medical Sciences and Research Center, Amrita University, Ponekkara, Cochin 682041, Kochi, India *Corresponding Author

Amrutha PB

Post graduate student MSC MLT, Amrita Institute of Medical Sciences and Research Center, Amrita University, Ponekkara, Cochin 682041, Kochi, India

Anu Catherine Jacob

Lecturer, Amrita Institute of Medical Sciences and Research Center, Amrita University, Ponekkara, Cochin 682041, Kochi, India

ABSTRACT

The diagnostic assessment of problematic prostatic pathology is supported by immunohistochemistry. The loss of tissue on further sectioning of paraffin block, can be addressed by double or multiplex immunostains on a single tissue section. We evaluated in this study the utility of double immunostains of p63 and AMACR in diagnostic assessment of prostatic specimens. The absence of p63 basal marker in 100% along with intense diffuse cytoplasmic or apical expression of AMACR in 88% was noted. The overall success of 98% with double immunostains in our laboratory illustrates that it is a simple assay to perform and is an appropriate advancement in the molecular era with every bit of tissue is precious.

KEYWORDS : prostate double immunohistochemistry

Introduction

Immunohistochemistry plays a vital ancillary role in diagnosis of prostatic adenocarcinoma in difficult cases of small acinar proliferation. The diagnosis is aided by immunostaining for basal cell markers such as p63, cytokeratin 5/6 (CK 5/6), and high molecular weight cytokeratin (CK HMW) as well as prostate-'specific' markers like alpha-methylacyl coenzyme A racemase (AMACR).¹⁻³ The usual practice is to use basal cell markers and AMACR separately on two sequential sections and then co-relate finding with the tissue morphology in the area of concern.

With the ever increasing demand on the need of tissue for further ancillary tests especially molecular tests, the demand on conservation of tissue without compromising diagnosis arose. The double immunohistochemistry was an able enabler towards this cause.^{6,7} It exploited either presence of antigens on different cells or the co expression on different areas (nuclear, cytoplasm or membrane) within same cell. Also even co-localisation expression pattern (mixed color pattern) is possible using two different chromogens with high color contrast.^{8,9} The double immunostain basically requires two different visualization techniques with no cross reactivity. The antibodies can be applied either simultaneously or sequentially.

In this study we aimed to use double immunohistochemical staining for p63 and AMACR antibodies, which are applied simultaneously on one tissue section. This was a change from our routine practice and we wanted to assess the performance of double antibody immunohistochemistry in routine surgical pathology practice.

Materials And Methods

The study was a prospective study carried at the department over a period of six months. The study has been approved by the Institutional Review Board.

Study group

Method of collection of data was based on inclusion and exclusion criteria

Inclusion criteria – Archived cases of prostatic acinar adenocarcinoma in radical prostatectomy specimens received in histo pathology laboratory.

Exclusion criteria- Needle biopsies, TURP specimens, suprapubic prostatectomy specimens and all benign hyperplasia cases.

The study was conducted as a retrospective study. A total of archived

50 blocks of radical prostatectomy from 15 patients of acinar adenocarcinoma were used. The diagnosis of prostate cancer was established from examination of hematoxylin & eosin stained sections and slides which contained both benign and malignant glands were selected.

Immunohistochemical Analysis

Formalin fixed paraffin embedded tissue blocks were cut into 3µm sections, transferred to PLL coated glass slides. And treated with EDTA buffer pH9.0 in a pressure cooker and slides were stained using indirect staining procedure cocktail of 2 antibodies p63 and AMACR (Ready to use) were mixed and added (combined) and incubated for 2 hours. After rinsing with PBS buffer a mixture of secondary antibodies, Anti Rabbit polymer AP and Anti Mouse Polymer HRP were added and incubated for one hour. The reaction were detected by application of the anti mouse horse radish peroxidase substrate, DAB for 5-10 minutes for p63 and then antirabbit alkaline phosphatase substrate, fast red for 10 minutes for AMACR. After development of color the slides were rinsed and counter stain with hematoxylin and cover slipped with permanent mounting media.

Results

The staining was evaluated based on intensity, localization of antibodies in both malignant and benign areas, extend of staining, background staining and also the sensitivity and specificity of the stain.

All the selected blocks from the cases showed both benign and malignant areas. Out of the 50 cases studied only one case did not show any p63 or AMACR positivity in any area of the slide, thereby providing an overall success of 98% for the use of double immunohistochemical staining.

STAINING OF P63 AND AMACR IN BENIGN and MALIGNANT AREAS

Normally p63 only shows positivity in the surrounding myoepithelial cells in the benign glands and should be negative in malignant glands. In the present study most of the cases (71.4%) showed p63 positivity and AMACR negativity, followed by 36% showing p63 and AMACR positivity in benign areas.

In the present study 95.9% cases showed AMACR positivity and p63 negativity in the malignant glands and with 4% cases showing both AMACR and P63 negativity.

LOCALISATION of p63 and AMACR in prostate

In the present study it was found that P63 is a good stain because 49

cases (98%) showed nuclear positivity in myoepithelial cells of normal gland, 2 cases(4%) show non specific cytoplasmic staining. P63 was 100% negative in all malignant areas. AMACR was seen in malignant glands with 44 of the 49 cases (89.7%) showed cytoplasmic positivity of epithelial cells and 41 cases (83.6%) showed positive stain in apex.

Table1: localization of p63 and AMACR in normal and malignant areas within the epithelial and myoepithelial cells.

		LOCATION																			
		p63						AMACR													
		myoepithelial			epithelial			MYOEPITHELIAL			EPITHELIAL										
		NUCLEAR	CYTO	APICAL	NUCLEAR	CYTO	APICAL	NUCLEAR	CYTO	APICAL	NUCLEAR	CYTO	APICAL								
NORMAL	1	5	43	49	0	0	47	2	47	2	49	0	49	0	33	16	0	36	10	2	1
TUMOR	49	0	0	49	0	0	47	2	48	1	49	0	49	0	5	40	4	0	0	18	15

EXTEND OF STAINING

In the present study extend of staining was expressed as percentage of the benign and malignant areas. The area of coverage for p63 in the benign glands was 63.7 % and in the malignant areas p63 was 100% negative. Extend of staining of AMACR in benign areas were less than 10 % was 67.3 % of cases which is followed by 32.6 % cases which only showed 10-50 % . In malignant areas 51 % cases showed greater than 50 % extend of staining followed by 30.6% cases 10-50 % and 18.3 % less than 10 %.

INTENSITY OF STAINING

In normal glands 53% showed 2+ intensity for p63 stain followed by 44.8 % 1+ and 2 % showed 3+ intensity. AMACR showed 30.6 % 1+ and 2 % 2+ intensity in normal glands, this is because AMACR can also stain negative glands. In malignant glands P63 was 100% negative which indicate P63 is a strong marker for benign glands and AMACR showed 48.9% 3+ intensity followed by 38.7% 2+ intensity and 6 % 1+ intensity in malignant glands.

BACKGROUND STAINING

In this study p63 showed 38.7 %+ and 2% ++ background staining in normal areas and 10.2 % + intensity followed by 2% ++ intensity in tumor areas . For AMACR background was clean in 79.5 % in normal and 87.7 % in tumor areas.

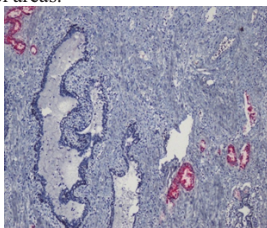


figure 1: Infiltrating tumor acini showing AMACR positivity with lack of p63. Normal prostatic glands show presence of p63 in basal cells on the left side. (H&E x40)

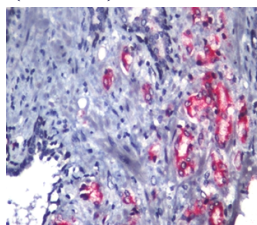


figure 2: Infiltrating tumor acini with dense cytoplasmic positivity and lack of p63 (H & E x400)

Discussion

The diagnostic sample are becoming smaller with less invasive sampling, there is a lot of demand on ancillary tests. Double immunohistochemistry helps address the challenges and even overcome the problem of performing serial sections. Our success rate of 98% with double immunohistochemistry using two antibodies p63 and AMACR proves the utility of the technique.

The traditional double staining techniques require two different visualization systems with no cross reactivity. The double enzymatic study can be achieved by simultaneous or sequential use of the two

antibodies.^{6,7} In our study we simultaneously applied the cocktail of p63 and AMACR to the tissue. This was followed by simultaneous mix of polymers anti mouse HRP and anti rabbit AP and finally sequential application of HRP substrate DAB and AP substrate fast red giving brown and red colors. In protocols using sequential antibodies, the order of primary antibodies is nuclear markers before membranous or cytoplasmic markers.

The most important hallmark of prostatic malignancy is the loss of basal cells. Immunohistochemistry strongly supports the diagnosis of malignancy by the absolute absence of basal cell markers. The known basal cell cytokeratins (CK 5/6, CK14, CK HMW) and p63 can be used as basal cell markers. AMACR (Alpha-methylacyl-CoA racemase) a positive marker of malignancy complements basal cell markers which stain negative in carcinomas.^{2,5}

In this study p63 was very sensitive and is seen to be positive in all normal and benign areas in the myoepithelial cells while not seen in any malignant areas. However in 2 cases aberrant epithelial nuclear and cytoplasmic positivity was seen in the normal and benign areas(4%). This has been seen as a nonspecific background cytoplasmic staining for p63 from more than the occasional laboratory, and could be attributed due to improper antibody titration or possible improper clone selection.¹⁰ The presence of p63 in malignant areas in occasional epithelial cells both in nucleus (2 cases) and in the cytoplasm (1 case) was noted. This aberrant diffuse expression of p63 in acinar adenocarcinomas create unique problems.¹¹ The total absence of other basal markers can help in these aberrant cases.¹²

AMACR was not be specific as it is expressed both in the benign and malignant areas in our study. However the intensity and degree of expression in terms of percentage of area of expression is lesser in the normal or benign areas compared to malignant areas. The study also showed more of apical positivity than cytoplasmic positivity in malignant areas as compared to benign areas. AMACR is a known to lack specificity and in about 20% of small foci of adenocarcinoma on needle biopsy be negative.^{13,14}

In practice a suspicious glandular focus that satisfies the criteria of carcinoma on histology and also is negative for basal cell markers can still be diagnosed as adenocarcinoma even though the AMACR reactivity is negative. A double immunohistochemistry with a positive and negative marker like in this study with p63 and AMACR helps demonstrate this concept and especially helps in decision making in small biopsies. The challenges of small biopsies of loss of tissue or more importantly an area of concern can be addressed by dual staining. In the era of molecular diagnostics tissue is the issue as not only diagnosis is important but also availability of tissue for further molecular analysis is of paramount importance.

Immunohisto chemistry no doubt has contributed to diagnostic pathology and also in prostate pathology. Double immunohisto chemistry in pathology in the new era of molecular pathology is a step in the right direction as it undoubtedly increases diagnostic certainty as well as conserves tissue for further analysis.

REFERENCES

1. Signoretti S, Waltregny D, Dilks J, et al. p63 is a prostate basal cell marker and is required for prostate development. *Am J Pathol* 2000; 157(6):1769-75.
2. Shah RB, Zhou M, LeBlanc M, Snyder M, Rubin MA. Comparison of the basal cell-specific markers, 34betaE12 and p63, in the diagnosis of prostate cancer. *Am J Surg Pathol* 2002; 26(9):1161-8.
3. Ollai BR, Kahane H, Epstein JI. Can basal cells be seen in adenocarcinoma of the prostate?: an immunohistochemical study using high molecular weight cytokeratin (clone 34betaE12) antibody. *Am J Surg Pathol* 2002; 26(9):1151-60.
4. Zhou M, Aydin H, Kanane H, Epstein JI. How often does alpha-methyl-acyl-CoA-racemase contribute to resolving an atypical diagnosis on prostate needle biopsy beyond that provided by basal cell markers? *Am J Surg Pathol* 2004; 28(2):239-43.
5. Browne T, Hirsch MS, Brodsky G, et al. Prospective evaluation of AMACR (P504S) and basal cell markers in the assessment of routine prostate needle biopsy specimens. *Hum Pathol*. 2004; 12:1462-1468.
6. Zhou M, Shah R, Shen R, Rubin MA. Basal cell cocktail (34betaE12 + p63) improves the detection of prostate basal cells. *Am J Surg Pathol* 2003; 27(3):365-71.
7. Molinie V, Fromont G, Sibony M, et al. Diagnostic utility of a p63/alpha-methyl-acyl-CoA racemase (p504s) cocktail in atypical foci in the prostate. *Mod Pathol*. 2004; 17:1180-1190.
8. Sanderson SO, Sebo TJ, Murphy LM, et al. Analysis of the p63-/methylacyl coenzyme A racemase immunohistochemical cocktail stain in prostate needle biopsy specimens and tissue microarrays. *Am J Clin Pathol*. 2004; 121:220-225.
9. Kiril Trpkov, Joanna Bartczak-McKay, BSc, MLT, and Asli Yilmaz, MD, FRCP. Usefulness of Cytokeratin 5/6 and AMACR Applied as Double Sequential Immunostains for Diagnostic Assessment of Problematic Prostate Specimens.
10. Gladell PP, Luthringer DJ, Amin MB. Best Practice in Diagnostic Immunohistochemistry Prostate Carcinoma and Its Mimics in Needle Core Biopsies. *Arch Pathol Lab Med* 2008; 132:1388-1396

11. Osunkoya AO, Hansel DE, Sun X, Netto GJ, Epstein JI. Aberrant diffuse expression of p63 in adenocarcinoma of the prostate on needle biopsy and radical prostatectomy: Report of 21 cases. *Am J Surg Pathol* 2008; 32(3):461-7.
12. Shah RB, Zhou M, LeBlanc M, Snyder M, Rubin MA. Comparison of the basal cell-specific markers, 34betaE12 and p63, in the diagnosis of prostate cancer. *Am J Surg Pathol* 2002; 26(9):1161-8.
13. Jiang Z, Wu CL, Woda BA, et al. Alpha-methylacyl-CoA racemase: a multi-institutional study of a new prostate cancer marker. *Histopathol* 2004; 45(3):218-25.
14. Zhou M, Aydin H, Kanane H, Epstein JI. How often does alpha-methylacyl-CoA-racemase contribute to resolving an atypical diagnosis on prostate needle biopsy beyond that provided by basal cell markers? *Am J Surg Pathol* 2004; 28(2):239-43.